

EFFECTS OF SORGHUM POLYPHENOLS ON IN VITRO STARCH
DIGESTIBILITY AND PROTEIN PROFILE OF WHEAT FLOUR TORTILLAS

A Thesis

by

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ABSTRACT

As incidences of diseases associated with dietary patterns increase in the United States, focus has been placed on improving nutritional quality of processed foods. Carbohydrates contribute the most calories in the American diet (55%), making starch-based foods a target for improvement. Tortillas are increasingly popular among American consumers, serving as a good target to address this problem. This study investigated the use of sorghum bran to increase polyphenols and dietary fiber in wheat flour tortillas and the effect on starch digestibility and protein profiles.

Refined wheat flour tortillas were substituted at 10%, 15%, and 25% (Baker's) with brans from wheat and white, brown, and black sorghum. Dough rheology, phenolic profile, starch digestibility, and protein profiles were evaluated after dough formation, hot pressing, baking, and over 14 days of storage.

Bran substitution affected dough rheology, producing rougher, stiffer, less extensible dough compared to the refined control. Processing, storage, and bran source significantly affected the phenolic profile of the tortillas. Total phenols, 3-deoxyanthocyanins, and proanthocyanidins (PA) decreased with processing and storage. Dough formation drastically decreased phenol content in brown sorghum bran dough compared to other treatments. Extractable high molecular weight PA also decreased dramatically after processing by 58 – 76% in brown sorghum bran tortillas. These tortillas had less rapidly digestible starch and more slowly digestible starch than other treatments at 25% substitution. Compared to the expected total dietary fiber (TDF),

sorghum brans doubled the formation of TDF (20 – 26%) as compared to wheat bran (11%). The largest increase was observed in brown sorghum bran tortillas.

In tortillas substituted at 25%, insoluble protein (IP) increased with baking and storage as extractable protein (EP) decreased. Within the EP fraction, soluble polymeric protein (SPP) decreased by 40 – 61% after baking. Brown sorghum bran dough contained significantly higher IP and lower SPP than other treatments; however, this effect was reduced after baking.

Sorghum brans provided polyphenols that interacted with protein and starch in wheat flour tortillas. PA and SPP largely contributed to these interactions, forming insoluble complexes that decreased tortilla digestibility and may positively benefit weight management.

DEDICATION

This thesis is dedicated to my family for supporting my decision to attend Texas A&M University and all of the encouragement they have given me while pursuing my M.S.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

The correlation between health and food consumption has been magnified in the United States as nutrition related diseases become more prevalent. Factors such as consuming too many calories and not enough dietary fiber, and associated consequences like obesity, diabetes, and other diseases associated with being overweight have increased the need to produce healthier processed foods.

Carbohydrates make up a large part of the American diet, contributing about 55% of the total calories (USDA, 2012); therefore, starch-based foods such as baked goods and snacks may serve as a good target to address nutrition and health. According to 2013 Tortilla Industry Association data, tortillas are an increasingly important baked product, and their share in the baked goods market has increased 9% annually since 1996. Tortillas were a \$12 billion market in 2012 in the United States, with wheat tortillas accounting for roughly half the market share. Tortillas are perceived as healthy and convenient, making them desirable to consumers; however, there is still limited diversity in wheat flour tortillas as more than 85% are prepared from refined wheat (TIA, 2013). With little variety in the current market and popularity among consumers, there is opportunity for tortilla innovation to address health concerns while satisfying consumer demands.

Consumer interest in individual nutrient claims such as whole grain and antioxidants provides an opportunity for product innovation in the flour tortilla market. Adding sorghum to wheat flour tortillas can address these areas of interest. Although traditionally used for feed in the United States, grain sorghum is experiencing an increase in food applications due to its status as an “ancient grain” and use as a gluten-free grain ingredient. Phenolic compounds in sorghum bran have beneficial health properties such as antioxidant, anti-inflammatory, and anticancer activity (Awika and Rooney, 2004; Yang et al., 2012). Condensed tannins present in some varieties of sorghum have been demonstrated to affect starch digestibility in a manner that could be beneficial in combating diseases associated with weight management such as obesity and diabetes (Barros et al., 2012).

However, the fate of these compounds in the matrix of processed foods is not well understood. Potential interactions with matrix constituents, such as starch and protein, and the effects of processing and storage should be investigated further in order to use sorghum to deliver bioactive polyphenols in a food system. Tortillas may serve as a vehicle to demonstrate the effects of sorghum bran in baked goods and its potential health benefits.

Research objectives

- 1) Evaluate the changes in sorghum polyphenols during processing and storage of refined wheat flour tortillas.
- 2) Evaluate the effect of sorghum bran substitution on *in vitro* starch digestibility in processed tortillas.

- 3) Evaluate the potential interactions of proteins with sorghum bran polyphenols during processing and storage of tortillas.

Literature review

Whole grains and health

With the increasing occurrence in the United States of diseases associated with weight management, focus has been placed on dietary intervention as an important tool for prevention and management of these diseases (CDC, 2011; Ogden et al., 2012). The Dietary Guidelines for Americans (DHHS, 2010) emphasize diets rich in fruits, vegetables, lean meats and whole grains while remaining within caloric restrictions.

The association of whole grain consumption (in place of refined grains) with reduced risks for cardiovascular disease (Anderson et al., 1991; Tighe et al., 2010), certain types of cancer (Okarter and Liu, 2010), diabetes (Parker et al., 2013), and other chronic diseases has been attributed to nutrients, such as dietary fiber (non-starch polysaccharides), and non-nutrients like bioactive phenols that are present at higher concentrations in whole grain.

Dietary fiber (DF) and resistant starch (RS) components concentrated in the bran portion of grains provide a different carbohydrate profile than the endosperm alone, contributing to the proposed mechanisms for their protective effects. DF and RS are not digested in the small intestine, but can be fermented in the colon, providing substrate for the production of short chain fatty acids (SCFA) (Ehle et al., 1982; Englyst and McFarlane, 1986), which have been shown to have protective effects against the development of colon cancer (Dolara et al., 2002).

The phytochemical composition of grains and their potential benefit when consumed as part of the human diet have been widely investigated (Okarter and Liu, 2010; Awika et al., 2004; Dykes and Rooney, 2007). Phenolic acids can be released from dietary fiber when fermented in the colon (Vitaglione et al., 2008) and play a role in chemoprevention. Phenolic compounds have also been shown to interact with starch to produce RS (Khan et al., 2013; Barros et al., 2012).

Potential of sorghum as a food ingredient

Sorghum (*Sorghum bicolor*) is a grain and forage crop with applications in the production of food and feed, sugar, and ethanol. Drought resistance and efficient energy conversion make sorghum a viable alternative crop (Rosenow et al., 1983; NSP, 2013). These agronomic advantages in combination with the composition of sorghum, such as the presence of high levels of polyphenolic compounds and absence of gluten proteins, contribute to sorghum's potential as a food ingredient (Taylor et al., 2013).

Natural variation among sorghum varieties contributes to diverse polyphenolic composition. Phenolic acids (Hahn et al., 1983), 3-deoxyanthocyanins (pigments) (Awika et al., 2004), and proanthocyanidins (tannins) (Strumeyer and Malin, 1975) have been identified and quantified in grain sorghum. Because of these characteristics, sorghum has been proposed as a functional ingredient to increase the phenolic content of breads (Hague, 2001), pasta (Khan et al., 2013), and flat breads (Yousif et al., 2012), as an alternative grain for use in gluten free breads (Schober et al., 2005; Boswell, 2010), and a natural antioxidant to retard lipid oxidation in ground beef products (Hemphill, 2006).

Composition of sorghum polyphenols

Sorghum phenolic acids

Like other cereal grains (Guo and Beta, 2013), phenolic acids in sorghum may be bound to the cell wall components (Hahn et al., 1983); however, new evidence suggests that glycerol ester derivatives of phenolic acids are predominant in sorghum (Yang et al., 2012). Phenolic acids and their antioxidant properties are believed to contribute to the protective effects of whole grains against chronic diseases (Slavin et al., 1997).

Sorghum anthocyanins

Anthocyanins are a group of pigments that are widely distributed among plant species. They may be provided in the diet by a variety of fruits, vegetables, and grains. Anthocyanins are of interest in foods because they exhibit various bioactive properties and function as a natural food colorant, providing red-blue-purple hues (Wrolstad, 2004). However, the use of these pigments is limited by their stability under processing conditions such as pH and temperature (Stitzing et al., 2002; Wrolstad, 2004).

Some sorghum varieties are unique in their anthocyanin composition in that they contain high levels of 3-deoxyanthocyanins, a group of anthocyanins less common than those found in fruits and vegetables (Awika et al., 2004). Luteolinidin, apigeninidin, and their derivatives are the most prevalent sorghum 3-deoxyanthocyanins (Figure 1). The 3-deoxyanthocyanins have been shown to exhibit greater stability to pH change as compared to anthocyanins (Awika et al., 2004); therefore, sorghum may be of interest as a source to supply natural food colorants. Sorghum 3-deoxyanthocyanins have also been shown to have a protective effect against proliferation of cancer cells (Yang et al., 2009).

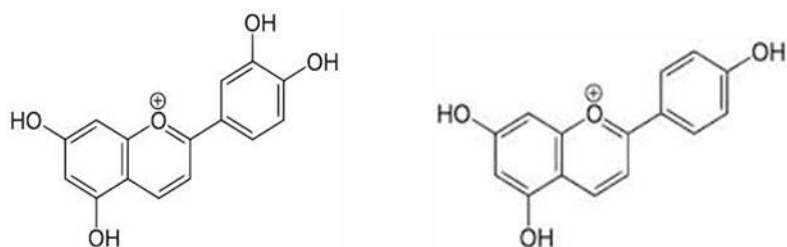


Fig. 1. Sorghum 3-deoxyanthocyanins luteolinidin (left) and apigeninidin (right).

Sorghum proanthocyanidins

Tannins are complex secondary metabolites found in plants. Among grains, they have been found in some cultivars of sorghum and barley (Strumeyer and Malin, 1975). Tannins may be classified as hydrolyzable or condensed (proanthocyanidins) (Figure 2), which are further characterized by their molecular weight distribution. Tannins are only found in sorghums with a pigmented testa layer. Sorghum may be classified by the presence of tannins: Type I, pigmented testa is absent and contains no extractable tannins; Type II, tannins extractable in 1% HCl in methanol; Type III, some tannins also extractable in unacidified methanol (Asquith et al., 1983).

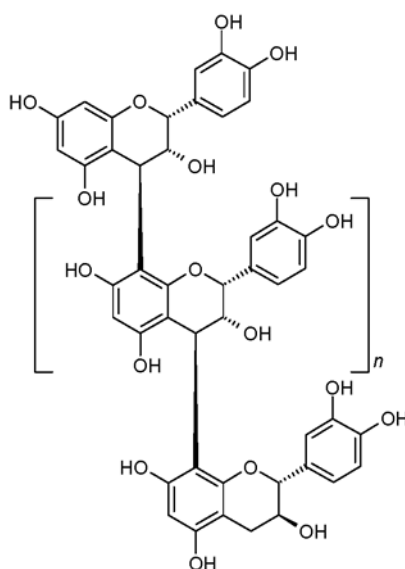


Fig. 2. General polymeric structure of condensed tannins.

Tannins have been shown to interact with several nutrients, specifically protein and carbohydrates (Asquith and Butler, 1986; Rooney and Pflugfelder, 1986). The role of tannins from sorghum in binding nutrients and their influence on digestibility is of particular interest and has been widely investigated. Biological activity is dependent not only on the content, but also the composition of tannins (Kaufman et al., 2012; Asquith and Butler, 1986) as well as the characteristics of the constituents with which they are interacting. For example, tannin-protein affinity is inversely related to molecular weight of the protein. Tannins also have a higher affinity for proteins rich in proline residues and precipitation is favored near the isoelectric point of the protein (Hagerman and Butler, 1981).

Nutritional importance of starch

Starch comprises a significant portion (80 – 90%) of the food carbohydrates supplied by the human diet (Englyst and Hudson, 1996). Food carbohydrates are generally classified by their chemical and structural properties such as branched amylopectin or mostly linear amylose. However, different physiological behavior of starches during the process of digestion requires characterization beyond their chemical classifications. Starch digestion products are therefore categorized based upon the rate and extent of digestion (Englyst et al., 2000). These categories include rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS). RDS and SDS make up the fraction of starch completely digested in the small intestine. RS is defined as the starch, and degradation products thereof, not digested in the small intestine and pass into the colon (Englyst and Cummings, 1990). Because RS is available for metabolism in the large intestine, the role it plays in nutrition is more similar to the non-starch polysaccharides (NSP) that make up dietary fiber than RDS and SDS.

The rate and extent of starch digestion has been correlated to glycemic response. SDS has a moderating effect on the postprandial glucose response as compared to the rapid peak in blood glucose level associated with consumption of RDS (Lehmann and Robin, 2007).

Numerous benefits of consuming RS as part of the diet have been identified (Annison and Topping, 1994; Englyst et al., 2000; Fuentes-Zaragoza, 2010; Brown, 2004). Addition and formation of RS in foods may reduce the caloric density of carbohydrate foods while increasing dietary fiber (Eerlingen and Delcour, 1994). In

rodents, RS has also been shown to upregulate expression of GLP-1 and PYY, hormones associated with satiety (Zhou et al., 2008). RS may also serve as a prebiotic ingredient or function as a symbiotic with a probiotic (Thompson et al., 2011). These potential health benefits serve as a driving force in research in the area of increasing yields of SDS and RS in processed foods.

Potential tannin-starch interactions

Decreased feed efficiency in animals has been associated with diets containing high-tannin sorghums due to tannin interactions with protein (Cousins et al., 1981; Robbins et al., 1987). Tannin-protein interactions have been extensively studied; however, there is less evidence supporting the interactions between tannins and carbohydrates and their impact on starch digestibility.

Tannic acid decreased *in vitro* starch digestibility of legume, sweet potato, potato (Deshpande and Salunkhe, 1982), and wheat starches (Thompson and Yoon, 1984). Tannin extracts from sorghum have been shown (*in vitro*) to increase RS formation when cooked with starch, specifically amylose (Barros et al., 2012). Barros et al. (2014) also showed interactions between amylose and proanthocyanidins were affected by the molecular weight of the proanthocyanidins, with those of higher molecular weight binding more strongly with amylose when compared to those of lower molecular weight.

Reduced starch digestibility due to tannin-starch interactions could decrease the caloric density of foods and potentially contribute to weight management. The benefits of SDS and RS in the diet discussed previously in this section are cause for further research in the area of tannin-starch interactions in the matrix of processed foods.

CHAPTER II

EFFECTS OF SORGHUM POLYPHENOLS ON IN VITRO STARCH DIGESTIBILITY OF WHEAT FLOUR TORTILLAS

Introduction

The incorporation of whole grain and bran ingredients into refined products is justified by two key properties: their bioactivity (antioxidant, anti-inflammatory, anticancer, etc.) and their impact on digestibility of the finished product. However, when using functional ingredients to enhance the nutritional quality of a processed food, the changes the product undergoes as a result of processing and storage before reaching its end-use (consumer) must be taken into consideration.

In the case of phenolic compounds from cereal grains, processing may be beneficial or detrimental. Extractability and bioavailability of some phenolic compounds may increase as a result of exposure to heat and moisture during processing such as baking and extrusion (Gu et al., 2008) or as a result of the fermentation process used to produce some foods, such as in sourdough bread (Katina et al., 2005).

On the other hand, heat processing can destroy phenolic compounds. The products of thermal degradation may shift the profile of phenolic compounds by altering ratios of free and bound phenolic acids (El-Sayed and Rabalski, 2013) or the molecular weight distribution of procyanidins (Awika et al., 2003). Compounds that impart color may also be degraded and have an adverse effect on product quality perceived by the

consumer. The use of natural colorants may appeal to consumers; however, the color must show stability during food processing and handling.

Complexation of starch with certain phenolic compounds, such as condensed tannins, may also hinder the digestion of starch in the small intestine (Haralampu, 2000). Interactions with matrix constituents may modify the starch digestibility profile to create a product with a glycemic response more favorable in managing blood glucose levels and associated diseases.

Sorghum bran contains a high level of diverse polyphenols compared to other cereal brans. Phenolic acids, 3-deoxyanthocyanin pigments, proanthocyanidins, among others, are usually present to varying degrees depending on the sorghum variety. Because of the diversity in phenolic composition, the effect of different classifications of polyphenolic compounds on food processing can be readily investigated.

Tortillas have a perception of health and convenience and are increasing in popularity among consumers in the United States (TIA, 2013). Thus, they are an important vehicle to improve health attributes of food. Tortillas are produced using a short baking time (1-2 minutes); therefore, the adverse effects of heat processing on phenolic compounds should be minimal as compared to other baked goods such as bread.

The objective of this work was to determine how polyphenolic compounds, specifically proanthocyanidins, from sorghum bran affect the starch digestibility of wheat flour tortillas and how sorghum bran phenolic profile is altered during tortilla processing and storage.

Materials and methods

Materials

Three different types of sorghum bran were chosen for their diversity in polyphenolic composition. White sorghum (ATxArg-1/RTx436, TAMU, College Station, TX, 2008) was decorticated in a PRL mini-dehuller (Nutama Machine Company, Saskatoon, Canada) and separated (Model 6DT4-1, KICE Industries Inc., Wichita, KS) to yield 12% bran. Brown and black sorghum brans were commercial samples (Nu Life Market, Scott City, KS). Brown and black sorghum brans were chosen for their higher phenol content as compared to white sorghum bran, specifically proanthocyanidins in the brown bran and 3-deoxyanthocyanins in the black bran. Wheat bran (ADM, Decatur, IL) was used as a baseline comparison as it is the most commonly consumed whole grain in the United States. The wheat and white sorghum brans were further milled (WonderMill, The WonderMill Company, Pocatello, ID) to reduce the particle size so that they were more similar to the brown and black sorghum brans.

Product formulation

Flour tortillas substituted with wheat and sorghum brans at 10, 15, and 25% (Baker's percent) were used for this research (Table I). The tortilla flour blend was a 50:50 preparation of high gluten bread flour (C. H. Guenther and Son, Inc., San Antonio, TX) and pastry flour (Cargill Inc., Minneapolis, MN). Bran substituted tortillas were compared to a control made with 100% refined tortilla flour blend. Each batch of tortillas was prepared using 1000 g of flour blend and bran, adjusted to the respective substitution level. The tortilla formulation also included 15.0 g iodized salt (Morten Salt

Inc., Chicago, IL), 5.0 g sodium stearyl lactylate (Caravan Ingredients, Inc., Lenexa, KS), 4.0 g potassium sorbate (Caravan Ingredients, Inc., Lenexa, KS), 6.0 g sodium propionate (Niacet Corporation, Niagara Falls, NY), 6.0 g baking soda (Church and Dwight Co., Inc., Ewing, NJ), 5.8 g sodium aluminum sulfate (Budenheim USA, Columbus, OH), 3.3 g encapsulated fumaric acid (Balchem Inc., New Hampton, NY), 60.0 g all-purpose shortening (Cargill Inc., Minneapolis, MN) and distilled water.

Because sorghum is gluten free, the amount of gluten in the refined flour-bran blend was diluted with increasing bran substitution level. To correct for this, 12.0, 19.0, and 31.0 g of vital wheat gluten (Cargill Inc., Minneapolis, MN) were added to the 10, 15, and 25% substitution levels, respectively. These values were calculated based on the gluten content of the refined flour blend which was determined by the gluten handwashing method (method 38-10.01, AACC Int., 1999a).

Table I

Tortilla formulations

Ingredient (g)	Control	25% Substitution				15% Substitution				10% Substitution			
		WA ^a	WI	BR	BL	WA	WI	BR	BL	WA	WI	BR	BL
Flour^b	1000	750	750	750	750	850	850	850	850	900	900	900	900
Bran	0	250	250	250	250	150	150	150	150	100	100	100	100
Vital wheat gluten	0	31	31	31	31	19	19	19	19	12	12	12	12
Salt	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0
Sodium stearoyl lactylate	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Potassium sorbate	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Sodium propionate	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
Sodium bicarbonate	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
Sodium aluminum sulfate	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8
Fumaric acid	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3
Shortening	60	60	60	60	60	60	60	60	60	60	60	60	60
Distilled water	520	670	593	590	620	580	560	560	580	560	554	540	550
Mix time (min)	7	9	8	8	10	8	7	7	7	7	6	6	8

^a bran treatment: WA = wheat, WI = white sorghum, BR = brown sorghum, BL = black sorghum

^b flour is 50:50 high gluten bread flour:pastry flour

Tortilla processing

Dry ingredients were mixed with a paddle attachment (model A-200, Hobart Corporation, Troy, OH) for 2 minutes at speed 1 (low). Shortening was then incorporated into the dry ingredients at low speed for 3 minutes. Warm distilled water (~35 °C) was added and mixed at low speed for 1 minute using the hook attachment. Dough was formed by mixing at speed 2 (medium). The mixer bowl was jacketed by metal tubing circulating water at 70 °C.

After mixing, the dough was allowed to rest for 5 minutes in a proofing chamber (Model 57638, National Manufacturing Co., Lincoln, NE) at 32 °C and 60-70% relative humidity. The dough was then pressed onto a stainless steel dividing plate and divided and rounded (Duchess Divider and Rounder, Bakery Equipment and Service Co., San Antonio, TX) into 36 pieces, approximately 30 g each. The dough balls were allowed to rest for another 10 minutes in the proofing chamber under the conditions listed above.

The dough balls were hot-pressed (400 °F, 1150 psi, 1.4 sec) and baked in a three-tier gas oven (Model 0P01004-02, Lawrence Equipment, El Monte, CA) for approximately 40 seconds at 380-400 °F. Tortillas were cooled to room temperature on a three-tier conveyor (Superior Food Machinery, Inc., Pico Rivera, CA) and packaged in 2 mm polyethylene bags for storage.

Sampling

Samples were retained from 6 different processing stages: dough, hot-pressed but not baked, immediately after baking (day 0), and after 1, 7, and 14 days storage at room temperature. Dough, pressed, and Day 0 samples were flash frozen in liquid nitrogen.

Day 1, 7, and 14 samples were frozen after the respective days of room temperature storage. All samples were ground in a coffee grinder to reduce the particle size then dried for 2 hours in a hot air oven at 40-50 °C, ground with a UDY cyclone sample mill to pass through a 0.1 mm screen (Model 3010-030, UDY Corporation, Fort Collins, CO), and stored at -20 °C until analysis. Residual moisture content of the dried samples was determined by a moisture analyzer (Model HB43-S, Mettler-Toledo, LLC, Columbus, OH).

Dough properties

Subjective dough rheology

Subjective dough rheology was performed according to Seetharaman et al. (2000) and Alviola et al. (2008) for smoothness, softness, force to extend, extensibility, and press rating. All attributes were ranked on a 5 point hedonic scale (Table II).

Objective dough rheology

Objective dough rheology was measured by a TA.XT2 Texture Analyzer (Texture Technologies Corp., Scarsdale, NY/Stable Micro Systems, Godalming, Surrey, UK). Force to compress (N) (uniaxial compression; 70% strain; 10 mm/s) and stress relaxation time (s) (uniaxial compression; 70% strain; 120 s) of dough balls (~30 g) were determined (Barros, 2009).

Table II

Subjective dough rheology evaluation parameters

Score	Smoothness ^a	Softness ^b	Force to Extend ^c	Extensibility ^d	Press Rating ^e
1	very smooth	very soft	less force	breaks immediately	less force
2	<u>smooth*</u>	<u>soft</u>	<u>slight force</u>	some extension	<u>slight force</u>
3	slightly smooth	slightly hard	some force	<u>extension</u>	some force
4	rough	hard	more force	more extension	more force
5	very rough	very hard	extreme force	extends readily	extreme force

^a smoothness: appearance and continuity of the dough surface

^b softness: firmness of dough when compressed with fingers

^c force to extend: elasticity of the dough when pulled apart by hand

^d extensibility: length of dough extension when pulled apart by hand

^e press rating: force required to flatten dough

*desired score for each attribute

Polyphenolic composition

Defatted samples were used for all analyses that determined polyphenols. Dried, ground samples were defatted by extracting in hexanes (3:1 hexanes:sample) for 2 hours with shaking at room temperature. Samples were centrifuged, the supernatant was discarded, and the hexane extraction was repeated again. Defatted samples were air dried in a fume hood until all remaining hexanes had evaporated. The samples were then stored at -20 °C until used for analysis.

Total phenolic content

Total polyphenols were measured according to the Folin-Ciocalteu method (Kaluza et al., 1980). Samples were extracted in 1% HCl in methanol with shaking for 2 hours. Aliquots were diluted with distilled water and reacted with Folin-Ciocalteu reagent (0.4 mL) and ethanolamine (0.9 mL) for 20 minutes at room temperature. Absorbance at 600 nm was read on a UV-Vis spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). Results were expressed as mg gallic acid equivalents (GAE)/g based on a standard curve prepared with serial dilutions of gallic acid in methanol.

High performance liquid chromatography (HPLC) analysis

Polyphenols were also profiled and identified using an Agilent 1100/1200 HPLC system (Agilent Technologies, Santa Clara, CA). Phenolic acids and anthocyanins were analyzed following the method described by Awika et al. (2009) at wavelengths of 280, 330, 360, and 480 nm using a diode array detector. A reverse phase Luna 5u C18(2) column (150 x 4.6 mm, 5 µm, Phenomenex, Torrance, CA) was used. Samples were

extracted in 1% HCl in methanol (0.5 g in 5 mls) for 2 hours and filtered (0.45µm, nylon) before injection (10 µl). The mobile phase was composed of 1% formic acid in water (A) and 1% formic acid in acetonitrile (B). The elution gradient (B) over the 40 minute run time was: 0-3 min, 10% isocratic; 3-5 min, 10-18%; 5-10 min, 18-20%; 10-23 min, 20-26%; 23-25 min, 26-18%; 25-28 min, 28-40%; 28-30 min, 40-60%; 30-32 min, 60% isocratic; 32-34 min, 60-10%; 34-40 min, 10% isocratic. Flow rate was 1 mL/min.

Proanthocyanidins were analyzed and separated based on degree of polymerization according to Langer et al. (2011). A fluorescence detector with excitation and emission wavelengths of 230 and 321 nm, respectively, and a normal phase Develosil Diol column (250 x 4.6 mm, 5 µm particle size, Phenomenex, Torrence, CA) were used. Samples were extracted in 2% acetic acid in 70% aqueous acetone (1 g in 5 mls) for 2 hours and filtered (0.45µm, nylon) before injection (10 µl). The elution gradient (B) over the 83 minute run time was: 0-3 min, 7% isocratic; 3-57 min, 7-37.6%; 57-60 min, 37.6-100%; 60-67 min, 100% isocratic; 67-73 min, 100-7%; 73-83 min, 7% isocratic. Flow rate was 0.6 mL/min. Authentic standards were used to quantify proanthocyanidins with a degree of polymerization (DP) of 1 – 3. Quantitative data for proanthocyanidins with a DP greater than or equal to four were based on DP three peak responses. Results were corrected for molecular weight according to the degree of polymerization (DP).

In vitro starch digestibility

In vitro starch digestibility analyses were conducted using fully-baked tortilla samples (Days 0, 1, 7, and 14).

Total starch (TS)

The K-TSTA assay kit (Section (c): determination of total starch content of samples containing resistant starch, but no D-glucose and/or maltodextrins) from Megazyme (Megazyme International, Wicklow, Ireland) was used to determine TS (method 76-13.01, AACC Int., 1999b). Sample (~100 mg) and 80% (v/v) ethanol (0.2 mL) were added to a glass test tube and mixed on a vortex mixer. A magnetic stirring rod was added to each tube and the tubes were placed in an ice water bath before 2 M KOH (2 mL) was added to each tube with stirring. The tubes were removed after 20 minutes and 1.2 M sodium acetate buffer (8 mL) was added to each tube with stirring. Thermostable α -amylase (0.1 mL; 3,000 U/mL; Megazyme International, Wicklow, Ireland) and amyloglucosidase (0.1 mL; 3,300 U/mL; Megazyme International) were added to each tube. The tubes were incubated in a 50 °C water bath for 30 minutes with intermittent stirring on a vortex mixer. Samples were diluted to 100 mL with distilled water, centrifuged, and the supernatant was retained for analysis (method 76-13.01, AACC Int., 1999b). The K-GLUC (GOPOD format) assay kit from Megazyme (Megazyme International) was used for the determination of D-glucose using glucose oxidase based on absorbance at 510 nm as read on a UV-Vis spectrophotometer (Shimadzu Scientific Instruments).

Rapidly (RDS) and slowly digestible starch (SDS)

RDS and SDS were determined using the colorimetric version of the procedure described by Englyst et al. (2000) with the following modifications by Barros et al. (2012). The guar gum solution and glass beads were omitted. Dried and ground sample (0.200 g) was used for the analysis. The enzyme mixture was prepared from α -amylase (SKU A3176, Sigma Aldrich, St. Louis, MO) and purified amyloglucosidase (Cat. No. E-AMGDF, Megazyme International) at concentrations of ~300 U/mL and ~95 U/mL, respectively. The K-GLUC (GOPOD format) assay kit from Megazyme was used for the determination of D-glucose using glucose oxidase based on absorbance at 510 nm as read on a UV-Vis spectrophotometer (Shimadzu Scientific Instruments).

Resistant starch (RS)

The K-RSTAR assay kit from Megazyme (Megazyme International, Wicklow, Ireland) was used to determine RS (method 32-40.01, AACC Int., 2002).

Total dietary fiber (TDF)

TDF analysis was conducted according to method 985.29 (AOAC Int., 2012).

Statistical analysis

All procedures were performed in duplicate with the exception of total dietary fiber analysis. Means and standard deviations were determined using Microsoft Office Excel 2010 (Microsoft Corporation, Redmond, WA). Statistical Analysis Software (SAS) version 9.3 (SAS Institute, Cary, NC) was used for data analysis. Differences in treatments were determined at the 5% significance level ($\alpha=0.05$) using the general linear model (GLM) and least significant difference (LSD) for mean separation.

Results and discussion

Dough rheology

Subjective evaluation

Subjective rheological measurements relate to machinability and handling qualities of the dough. Dough smoothness and softness were significantly affected at each level of bran substitution ($p < 0.05$) (Table III). Smoothness was scored as a 2.0 for the control and ranged from 2.0 – 3.0 in the bran treatments, indicating less smooth dough was formed as a result of bran addition. Because smoothness is based on the surface of the dough, larger particle size of the brans as compared to refined flour contributed to scores trending towards rough. Softness was also scored at a 2.0 for the control and ranged from 2.0 – 3.3 in the bran treatments indicating bran substitution formed less soft dough. Extensibility, force to extend, and press rating are indicators of the strength of the dough. For each bran source, dough substituted at 25% required more force to extend and showed slightly less extensibility than the refined control (Table III).

Objective evaluation

Force to compress and stress relaxation time measured using the texture analyzer are empirical measures of viscoelastic properties of the dough. Force to compress is the force (N) required to compress the dough to 70% of its original height. It is an indicator of dough stiffness.

Stress relaxation time is defined as the time (s) it takes for the force to decay to 62.8% of the initial force needed to maintain 70% compression. It is a measurement of the dough's resistance to applied stress and is an indicator of elasticity and strength of

the gluten protein network. Generally, a weaker dough relaxes under the applied stress more readily and therefore has a shorter stress relaxation time.

The results for compression force follow similar trends observed in the dough strength as measured in subjective evaluation; however, significant differences were detected more frequently. Force to compress was significantly increased at the 15% and 25% substitution levels as compared to the control for all bran sources, indicating stiffer dough ($p < 0.05$) (Figure 3). A significant increase was also observed for the wheat and black sorghum brans when substituted at 10% ($p < 0.05$) (Figure 3). Use of whole grain and bran has been shown to increase the force to compress tortilla dough when compared to a refined flour control (Barros et al., 2009; Gritsenko, 2009). Fiber present in the bran portion of grains increases water absorption and contributes to a more rigid dough structure.

Addition of bran significantly decreased stress relaxation time as compared to the refined control for the 25% substitution level treatments, indicating this level of substitution of bran created less elastic dough ($p < 0.05$) (Figure 4). Among bran treatments, black sorghum bran tortilla dough deviated the most from the refined control, increasing force to compress to 263 N from 105 N and decreasing stress relaxation time from 1.6 s to 1.3 at the 25% substitution level. This suggests the black sorghum bran exhibited increased water absorption.

Overall, subjective and objective evaluation of tortilla dough showed bran substitution formed rougher, harder, less extensible and less elastic dough as compared to the refined control. These parameters of dough rheology have been correlated to

Table III

Effect of substitution level and bran treatment on subjective rheology evaluation scores of tortilla dough

	Control	25% Substitution				15% Substitution				10% Substitution			
		WA ^a	WI	BR	BL	WA	WI	BR	BL	WA	WI	BR	BL
Smoothness	2.0 c,B,y*	2.3 a,b,c	3.0 a	2.0 b,c	2.8 a,b	2.5 A	2.5 A	2.3 A,B	2.5 A	2.5 x	2.0 y	2.3 x,y	2.5 x
Softness	2.0 c,C,y	2.8 a,b	2.8 a,b	2.5 b	3.3 a	3.0 A	2.8 A,B	2.3 B,C	2.8 A,B	2.3 y	2.0 y	2.0 y	2.8 x
Extensibility	3.5	2.3	3.0	3.3	3.3	2.5	2.5	3.5	2.8	3.3	2.8	3.5	3.3
Force to extend	2.5 b	3.8 a	3.0 a,b	3.3 a,b	3.5 a,b	3.8	3.0	2.8	3.3	3.0	3.0	3.3	3.0
Press rating	2.3 B	3.0	3.0	2.8	3.0	3.8 A	3.0 A,B	2.8 A,B	3.0 A,B	3.0	2.5	2.8	3.0

^a bran treatment: WA = wheat, WI = white sorghum, BR = brown sorghum, BL = black sorghum*Values with the same letter in the same row for each substitution level are not significantly different ($p < 0.05$, LSD). Values were also compared to the control. No significant differences were detected in categories without letters.

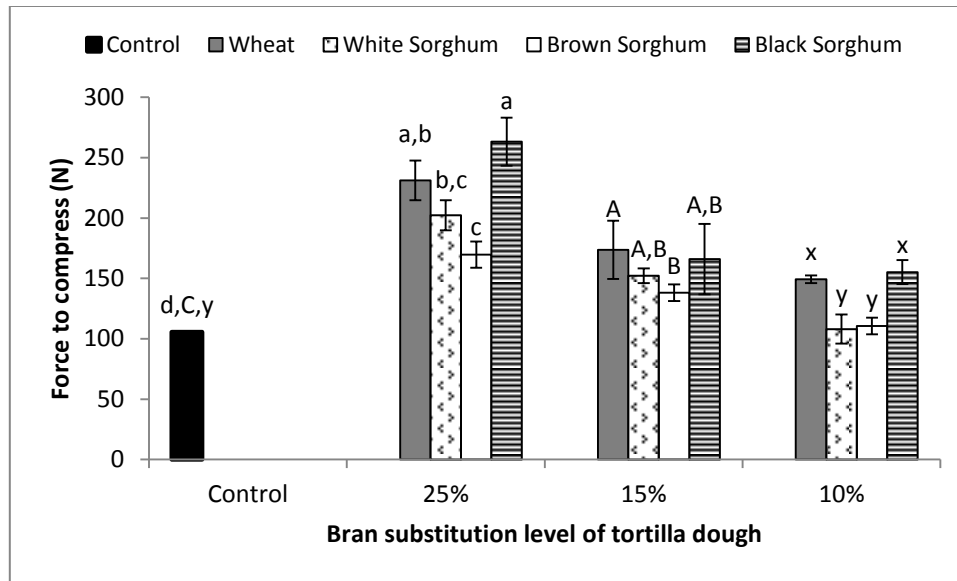


Fig. 3. Effect of bran substitution level on force to compress (N) tortilla dough. Bars with the same letter within the same substitution level are not statistically different ($p < 0.05$, LSD). Values were also compared to the refined control.

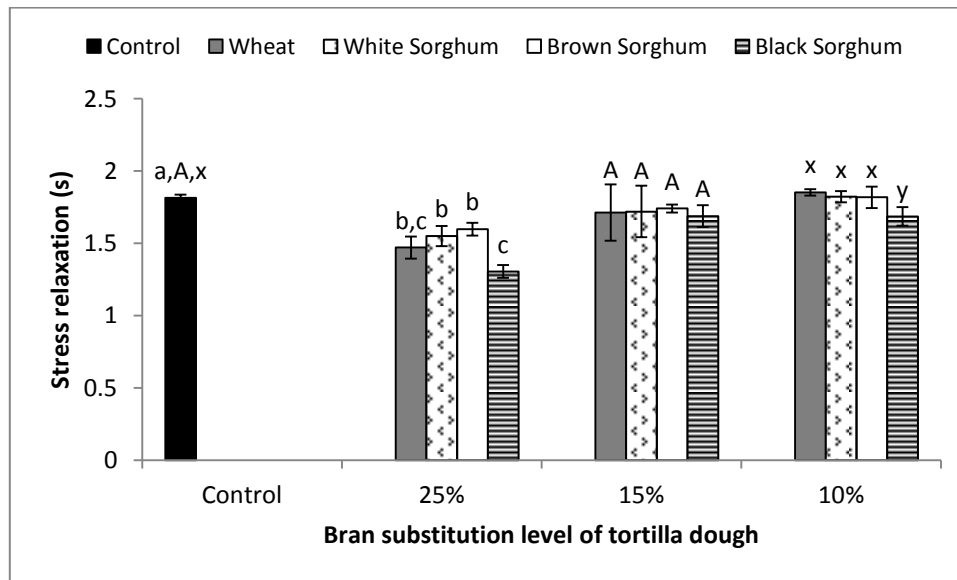


Fig. 4. Effect of bran substitution level on stress relaxation time (sec) of tortilla dough. Bars with the same letter within the same substitution level are not statistically different ($p < 0.05$, LSD). Values were also compared to the refined control.

characteristics of the finished tortilla product such as diameter, thickness, rollability, shelf life, and sensory attributes (Alviola et al, 2008; Barros et al., 2009; Jondiko, 2010).

Polyphenolic profile of tortillas

Total phenolic content

The amount of phenols present in the dry ingredient mix (dry mix) was calculated by multiplying the total phenol content of each bran by the percent of bran in the dry mix to account for the dilution effect of the other ingredients. In the treatments prepared with wheat and white sorghum bran, total phenol content initially increased upon dough formation then decreased with processing and storage ($p < 0.05$) (Figures 5 – 7). This may be attributed to increased extractability of phenols from the dough as compared to the bran in the dry mix. Phenolic acids are the predominant phenolic compounds in wheat and white sorghum, but are commonly bound to the cell wall (Hahn et al., 1983; Dykes and Rooney, 2007; Adom et al., 2003; Kim et al., 2006). Phenolic acids have been shown to be released from the bran cell wall matrix in the presence of moisture and heat, leading to increased extractability (Brennan et al., 2011). El-Sayed and Rabalski (2013) observed an increase in free phenolic acids in flat bread dough when prepared with whole grain einkorn wheat flour. On the other hand, dough formation decreased the total phenol content when brown and black sorghum bran were used ($p < 0.05$) (Figures 5 – 7). Due to nonspecificity of the Folin-Ciocalteu assay for phenols, other ingredients in the dry mix for the tortillas may have given false positives for phenols and contributed to the observed increase in phenols. This effect would have been masked by the large amount of phenols present in brown and black sorghum bran

as compared to wheat and white sorghum bran. Black and brown sorghum bran have higher levels of flavonoid compounds such as 3-deoxyanthocyanins and proanthocyanidins, respectively, that are likely to complex with gluten during dough mixing, resulting in decreased extractability and the observed decrease in total phenols upon dough formation.

At the 25% substitution level, the largest decrease in phenol content after processing and storage was observed in products prepared with brown sorghum bran (Figure 5). After dough formation, the brown sorghum bran treatment exhibited a 58% decrease in total phenols as compared to the starting material; the total loss from dry mix to Day 14 was 65%. For the black sorghum bran treatment, only 4 and 27% were lost as a result of dough formation and after 14 days of storage, respectively. Similar trends were observed at the 15 and 10% substitution levels, with 69 and 78% decreases in total phenols upon formation of dough, respectively (Figures 6 and 7). The large decrease in total phenols in dough prepared with brown sorghum bran compared to the other bran treatments suggests that tannins present in the brown sorghum bran interacted with proteins or starch upon dough formation to form unextractable complexes that could not be measured in this assay.

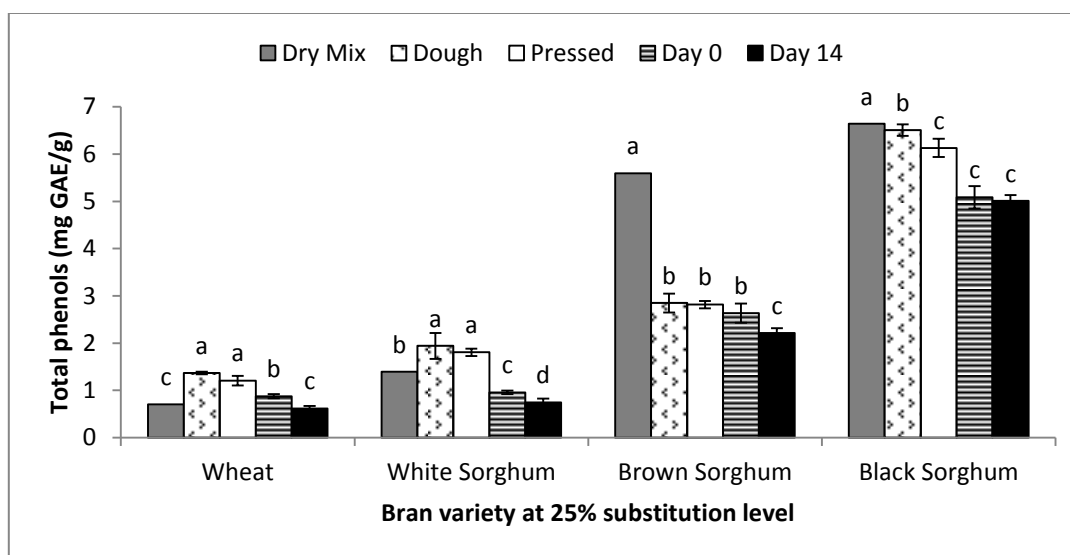


Fig. 5. Effect of bran source on total phenol (mg GAE/g) content of dry mix, dough, pressed, Day 0, and Day 14 tortillas substituted with bran at 25%. Bars with the same letter within the same bran treatment category are not statistically different ($p < 0.05$, LSD).

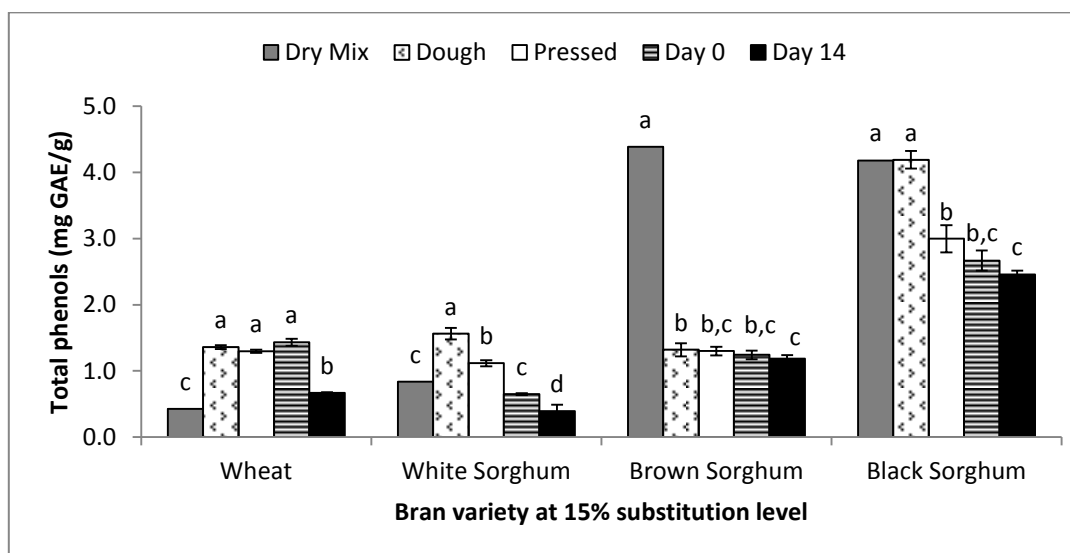


Fig. 6. Effect of bran source on total phenol (mg GAE/g) content of dry mix, dough, pressed, Day 0, and Day 14 tortillas substituted with bran at 15%. Bars with the same letter within the same bran treatment category are not statistically different ($p < 0.05$, LSD).

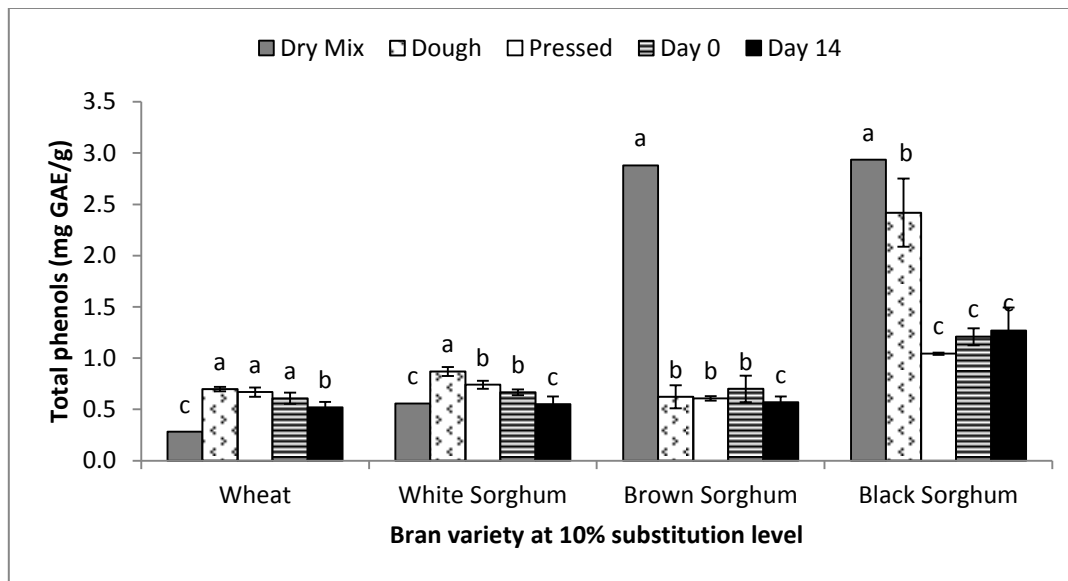


Fig. 7. Effect of bran source on total phenol (mg GAE/g) content of dry mix, dough, pressed, Day 0, and Day 14 tortillas substituted with bran at 10%. Bars with the same letter within the same bran treatment category are not statistically different ($p < 0.05$, LSD).

Samples made with black sorghum bran contained the highest levels of total phenols at each processing stage and storage time. Day 14 tortillas were compared at each substitution level to determine if the bran source affected the total phenol content in the final product. This comparison was chosen because, of the storage times evaluated in this research, the Day 14 tortillas are most representative of the end use product that reaches the consumer. For each substitution level, black sorghum bran had the highest phenolic content of Day 14 tortillas ($p < 0.05$) compared to substitution with brans from other sources (Figure 8). Brown sorghum bran provided significantly more total phenols than wheat and white sorghum in Day 14 tortillas when substituted at 25 and 15% ($p < 0.05$) (Figure 8).

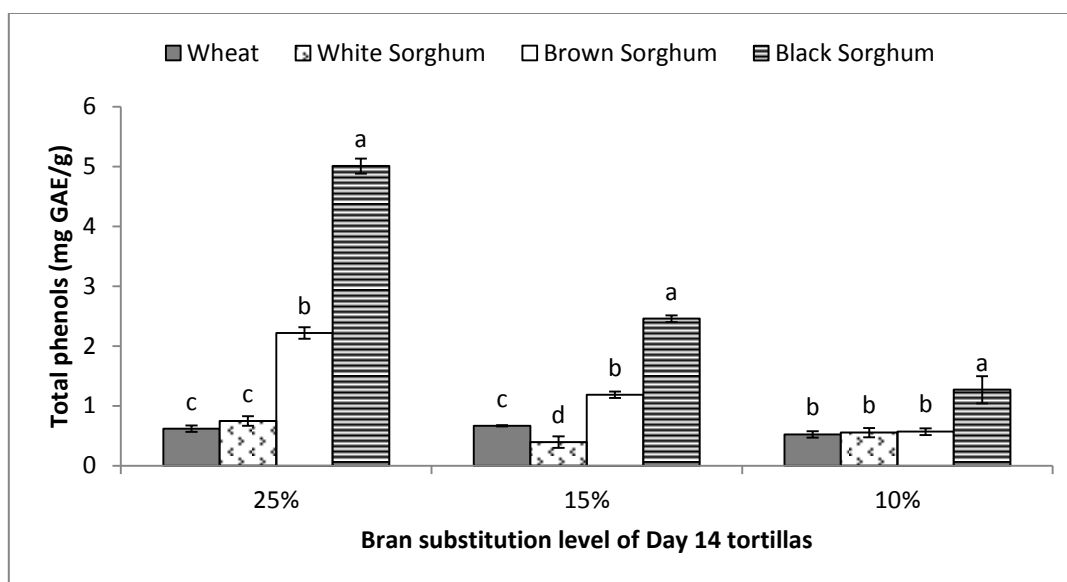


Fig. 8. Effect of bran source on total phenol (mg GAE/g) content of Day 14 tortillas substituted at 25, 15, and 10%. Bars with the same letter within the same substitution level are not statistically different ($p < 0.05$, LSD).

3-Deoxyanthocyanins

The 3-deoxyanthocyanins are a group of natural pigments found in sorghum that contribute favorable colors that consumers commonly associate with healthy foods. The ability to predict color stability of food products over processing and storage is necessary because color is a very important attribute to consumers and influences their purchasing decisions.

Luteolinidin, apigeninidin, and their methoxylated derivatives, such as 7-O-methyl-apigeninidin and 7-O-methyl-luteolinidin were the major 3-deoxyanthocyanins identified in black sorghum bran (Figure 9) and tortillas prepared from black sorghum bran (Figure 10). Luteolinidin was the major 3-deoxyanthocyanin identified in the samples, followed by 7-O-methyl-luteolinidin, apigeninidin, and 7-O-methyl-

apigeninidin. The major 3-deoxyanthocyanin pigments showed the same trends over processing and storage (Figures 11 – 14). Initially, decreases were observed as a result of dough formation and pressing as compared to levels in the dry mix. Concentration of the pigments then increased in Day 0 tortillas before decreasing again after 14 days of storage (Figures 11 – 14). As compared to the dry mix, Day 0 tortillas had 20 – 32% less luteolinidin, 1 – 9% less 7-O-methyl-luteolinidin, 44 – 63% less apigeninidin, and 17 – 28% less 7-O-methyl-apigeninidin. After 14 days of storage, tortillas had 20 – 33% less luteolinidin, 14 – 26% less 7-O-methyl-luteolinidin, 27 – 36% less apigeninidin, and 30 – 38% less 7-O-methyl-apigeninidin as compared to Day 0 tortillas. The magnitude of decrease in 3-deoxyanthocyanins increased with increasing level of bran substitution.

The initial decrease in 3-deoxyanthocyanins may be attributed to a change in extractability due to formation of the gluten protein network. Extractability of 3-deoxyanthocyanins may have improved with the degradation of proteins involved in the dough network upon baking. The decrease in 3-deoxyanthocyanins over 14 days of storage may also have been caused by decreased extractability of pigments as they formed insoluble complexes with proteins or retrograding starch.

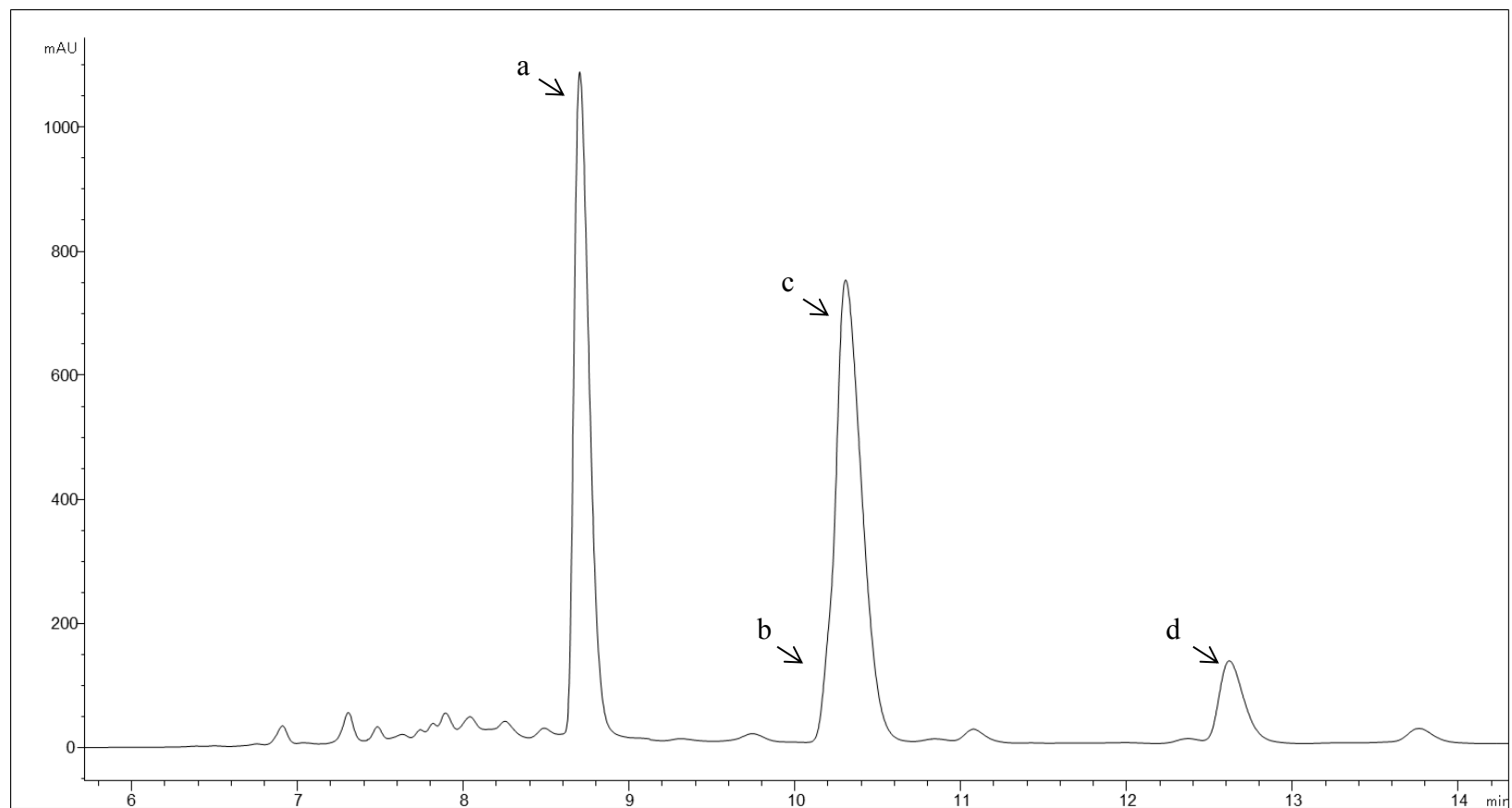


Fig. 9. Reverse phase HPLC phenolic profile of black sorghum bran at 480 nm showing major pigments luteolinidin (a), apigeninidin (b), 7-O-methyl-luteolinidin (c), and 7-O-methyl-apigeninidin (d).

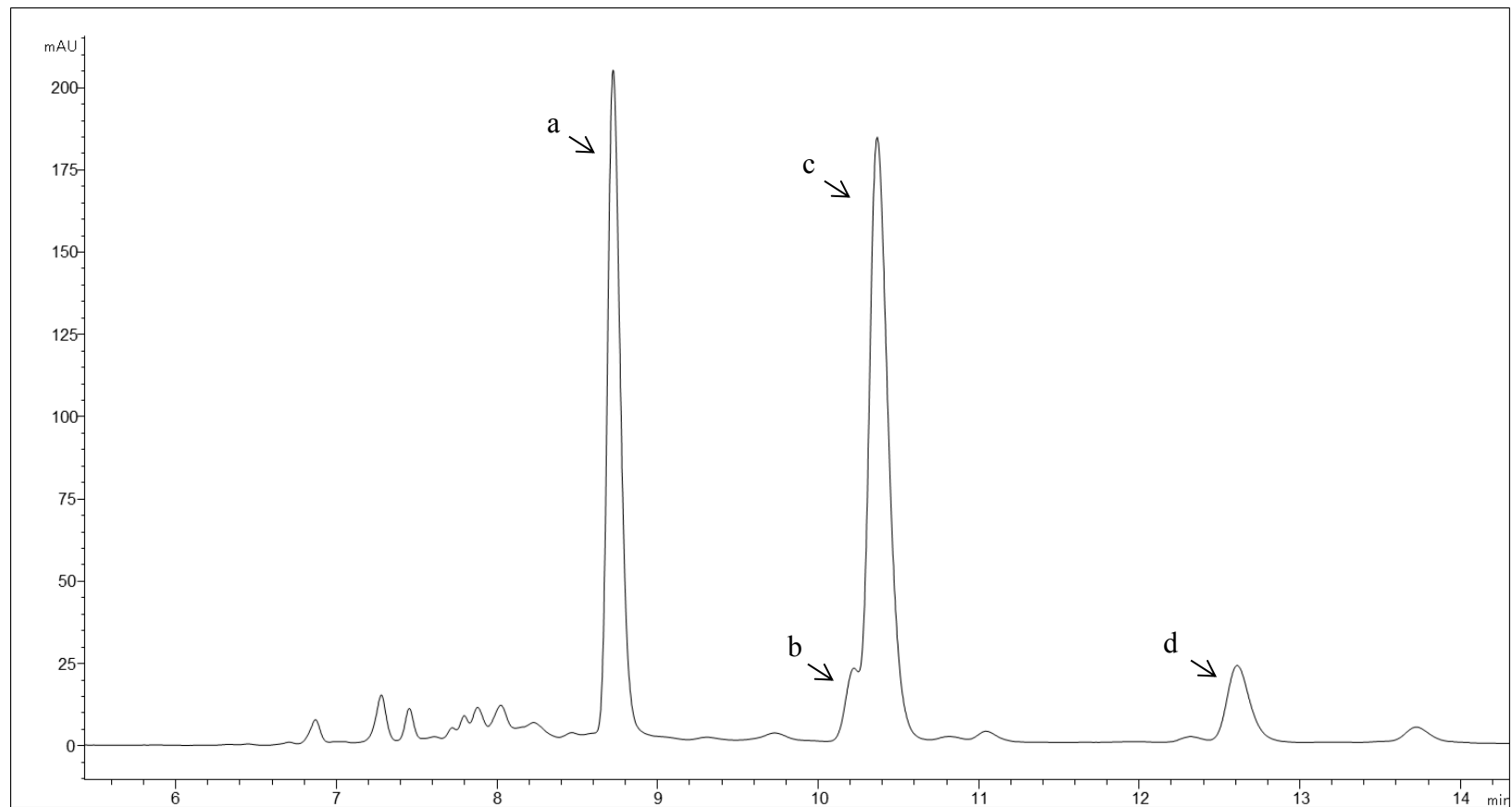


Fig. 10. Reverse phase HPLC phenolic profile of Day 0 tortillas prepared with black sorghum bran substituted at 25% showing major pigments luteolinidin (a), apigeninidin (b), 7-O-methyl-luteolinidin (c), and 7-O-methyl-apigeninidin (d) at 480 nm.

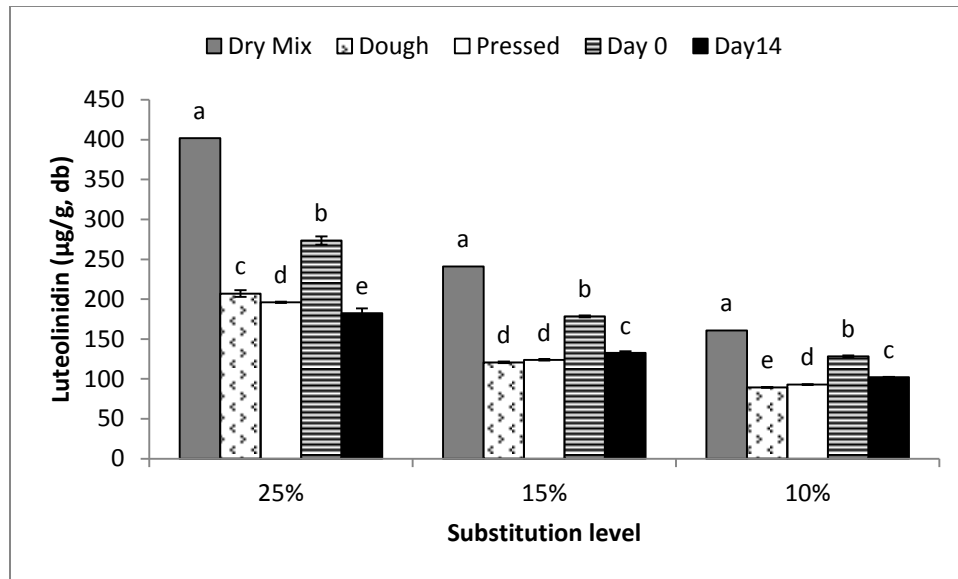


Fig. 11. Effect of black sorghum bran substitution level on luteolinidin content of dry mix, dough, pressed, Day 0, and Day 14 tortillas. Bars with the same letter within the same substitution level category are not statistically different ($p < 0.05$, LSD).

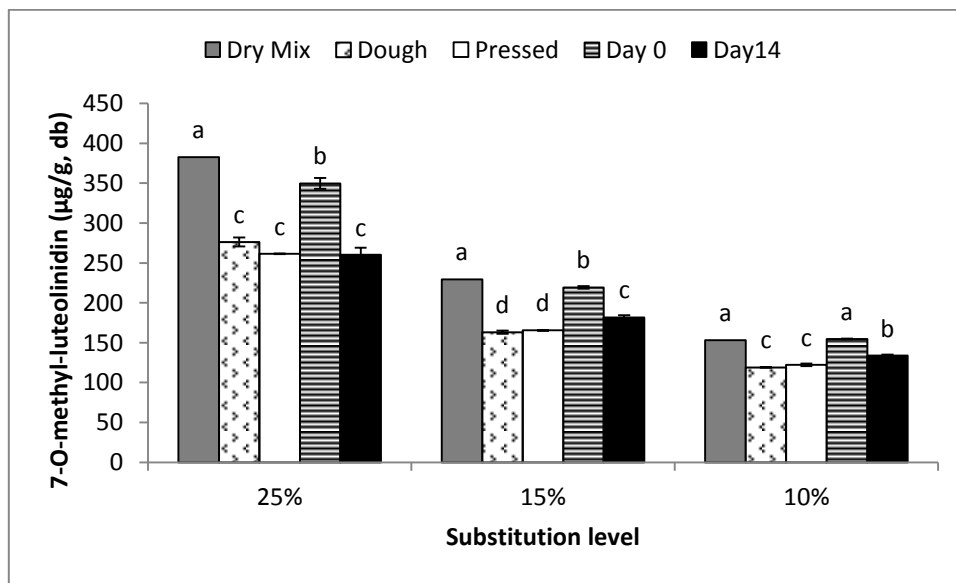


Fig. 12. Effect of black sorghum bran substitution level on 7-O-methyl-luteolinidin content of dry mix, dough, pressed, Day 0, and Day 14 tortillas. Bars with the same letter within the same substitution level category are not statistically different ($p < 0.05$, LSD).

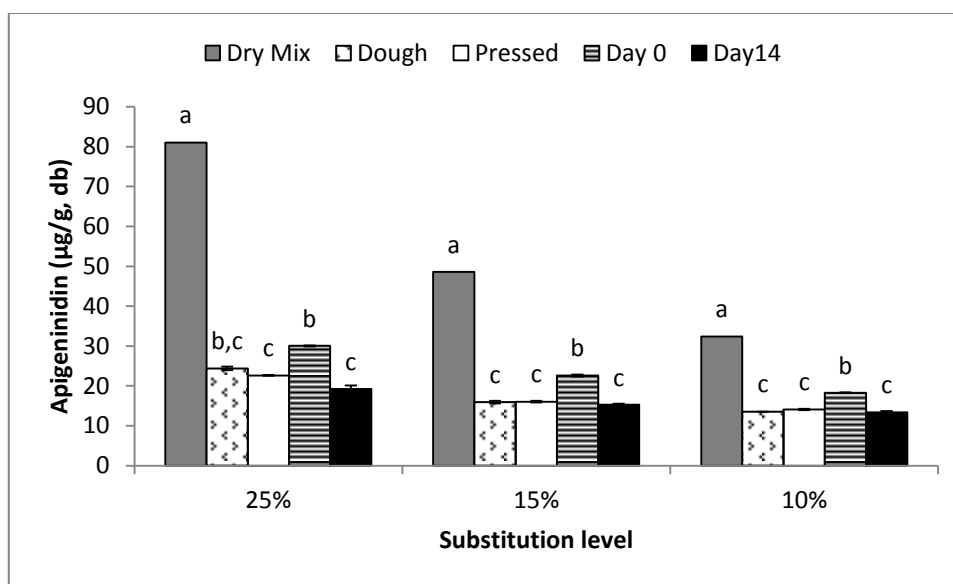


Fig. 13. Effect of black sorghum bran substitution level on apigeninidin content of dry mix, dough, pressed, Day 0, and Day 14 tortillas. Bars with the same letter within the same substitution level category are not statistically different ($p < 0.05$, LSD).

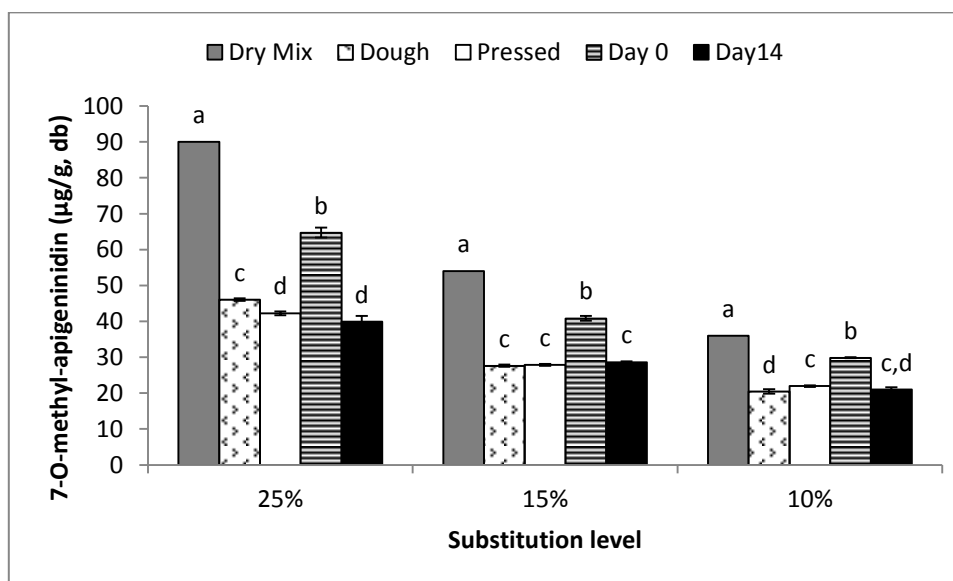


Fig. 14. Effect of black sorghum bran substitution level on 7-O-methyl-apigeninidin content of dry mix, dough, pressed, Day 0, and Day 14 tortillas. Bars with the same letter within the same substitution level category are not statistically different ($p < 0.05$, LSD).

Proanthocyanidin stability

Condensed tannins, or proanthocyanidins (PA), are present in sorghum varieties that contain a pigmented testa layer. PA from sorghum have been shown to reduce digestibility of proteins and starch in foods (Cousins et al., 1981; Robbins et al., 1987; Barros et al., 2012), making them of interest in this research.

Dry mixes for the 25, 15, and 10% brown sorghum bran substitution levels contained 2122, 1273, and 849 mg/g total extractable PA, respectively, as determined by HPLC (Tables IV-VI). Total extractable PA, especially high molecular weight PA, dramatically decreased with processing and storage over 14 days (Figures 15 – 18). The largest decrease was observed in PA with DP>10 (P) as a result of dough formation. Dough contained 781 ± 240 (25%), 297 ± 33.4 (15%), and 178 ± 11.6 (10%) mg/g PA (Tables IV-VI). When compared to the dry mix, this equals a percentage decrease of 58.2, 73.5, and 76.1%, respectively. The large change in high molecular weight PA extractability suggests condensed tannins are forming insoluble complexes with components in the dough such as starch or protein.

Formation of insoluble starch-PA complexes may slow down or inhibit digestion of the starch involved in the complex. Barros et al. (2012) showed that PA from sorghum extracts bound with starch, specifically amylose, resulting in the formation of resistant starch (RS).

Numerous studies (Asquith and Butler, 1986; Rooney and Pflugfelder, 1986; Cousins et al., 1981; Robbins et al., 1987) have shown a reduction in protein digestibility when protein-PA complexes form. If PA form complexes with proteins, they will be

unavailable to interact with starch, decreasing the impact PA have on starch digestibility as reported in a pure starch system (Barros et al., 2012). Functionality and degree of interaction depends on tannin content, as well as composition, and affinity of tannins to the non-tannin components (Kaufman et al., 2013, Elkin et al., 1996; Barros et al., 2014)

The observed decrease in PA likely explains the large decrease in extractable phenols in dough prepared with brown sorghum bran compared to other bran sources (Figures 5 – 7).

In vitro starch digestibility

Effect of bran substitution on total starch (TS)

TS contents of the brans ranged from 14.49 – 37.95% (db) (Figure 19). TS in Day 0, 7, and 14 tortillas substituted at 25% and the refined control ranged from 53.08 - 65.95% (db) (Figure 20). TS in Day 0, 7, and 14 tortillas substituted at 15% and the refined control ranged from 56.84 – 67.14% (db) (Figure 21). As expected, the addition of bran significantly decreased TS as compared to the refined control at 25% and 15% substitution levels ($p < 0.05$) (Figures 20 and 21). The observed decrease in TS occurred as a result of dilution of the starch by the bran added into the system. Because each bran contributed a different amount of starch to its respective products (Figure 19), subsequent starch readings, such as RDS and SDS, were reported as a percentage of TS of the final product on a dry basis.

Table IV

Extractable proanthocyanidin content^a of 25% brown sorghum bran tortillas

DP ^b	Bran	25% Substitution				
		Dry Mix	Dough	Pressed	Day 0	Day 14
1	47.3	11.8	25.1 ± 0.6	24.0 ± 0.2	18.7 ± 1.0	19.5 ± 0.4
2	84.9	21.2	20.5 ± 0.3	24.0 ± 0.3	24.8 ± 0.1	22.7 ± 0.3
3	35.2	8.8	3.4 ± 0.0	3.4 ± 0.0	4.6 ± 0.1	3.7 ± 1.1
4	50.8	12.7	7.1 ± 0.1	7.8 ± 5.6	13.1 ± 7.2	11.0 ± 0.1
5	45.0	11.3	7.4 ± 0.0	5.7 ± 1.3	10.9 ± 0.1	ND
6	47.7	11.9	16.5 ± 0.4	ND	ND	ND
7	172	43.0	16.1 ± 1.1	ND	10.3 ± 0.3	ND
8	210.4	52.6	ND ^d	ND	ND	ND
9	149.5	37.4	ND	ND	ND	ND
10	178.9	44.7	16.2 ± 3.1	ND	ND	ND
P ^c	7467.0	1866.8	780.9 ± 239.7	452.7 ± 23.8	475.7 ± 24.8	347.0 ± 6.9
Total	8488.6	2122.2	893.2 ± 243.8	520.4 ± 24.5	552.9 ± 23.7	407.3 ± 9.7
<u>Total</u>						
% change from previous process			-57.9	-41.7	6.2	-26.3
% change from dry mix						-80.8
% change from dough						-54.4
<u>P</u>						
% change from previous process			-58.2	-42.0	5.1	-27.1
% change from dry mix						-83.6
% change from dough						-55.6

^a mean ± standard deviation expressed as mg catechin equivalent/g (db) as determined by normal phase HPLC; corrected for molecular weight

^b DP, degree of polymerization

^c DP > 10

^d ND, not detected

Table V

Extractable proanthocyanidin^a content of 15% brown sorghum bran tortillas

DP ^b	Bran	15% Substitution				
		Dry Mix	Dough	Pressed	Day 0	Day 14
1	47.3	7.1	18.4 ± 0.0	18.5 ± 0.3	16.1 ± 1.2	16.1 ± 0.7
2	84.9	12.7	18.6 ± 0.5	18.3 ± 0.1	17.9 ± 0.2	16.1 ± 0.4
3	35.2	5.3	1.9 ± 0.4	ND	2.4 ± 0.0	ND
4	50.8	7.6	ND ^d	7.9 ± 0.0	6.9 ± 5.4	ND
5	45.0	6.8	ND	ND	ND	ND
6	47.7	7.2	ND	ND	ND	ND
7	172	25.8	ND	ND	ND	ND
8	210.4	31.6	ND	ND	ND	ND
9	149.5	22.4	ND	ND	ND	ND
10	178.9	26.8	ND	ND	ND	ND
P ^c	7467.0	1120.1	296.9 ± 33.4	251.2 ± 11.6	260.3 ± 13.4	214.0 ± 7.5
Total	8488.6	1273.3	335.5 ± 33.2	295.9 ± 11.9	303.6 ± 7.0	249.7 ± 2.3
<u>Total</u>						
% change from previous process			-73.7	-11.8	2.6	-17.8
% change from dry mix						-80.4
% change from dough						-25.6
<u>P</u>						
% change from previous process			-73.5	-15.4	3.6	-17.8
% change from dry mix						-83.2
% change from dough						-27.9

^a mean ± standard deviation expressed as mg catechin equivalent/g as determined by normal phase HPLC; corrected for molecular weight

^b DP, degree of polymerization

^c DP > 10

^d ND, not detected

Table VI

Extractable proanthocyanidin^a content of 10% brown sorghum bran tortillas

DP ^b	Bran	10% Substitution				
		Dry Mix	Dough	Pressed	Day 0	Day 14
1	47.3	4.7	14.6 ± 0.8	14.3 ± 0.3	13.9 ± 0.5	13.7 ± 0.2
2	84.9	8.5	19.6 ± 0.0	19.9 ± 0.1	13.9 ± 1.6	11.7 ± 0.0
3	35.2	3.5	ND ^d	ND	ND	ND
4	50.8	5.1	ND	6.3 ± 0.0	7.8 ± 0.3	9.7 ± 2.6
5	45.0	4.5	ND	ND	ND	ND
6	47.7	4.8	ND	ND	ND	ND
7	172	17.2	ND	ND	ND	ND
8	210.4	21.0	ND	ND	ND	ND
9	149.5	15.0	ND	ND	ND	ND
10	178.9	17.9	ND	ND	ND	ND
P ^c	7467.0	746.7	178.4 ± 11.6	166.7 ± 4.6	155.4 ± 13.4	129.7 ± 3.3
Total	8488.6	848.9	213.0 ± 13.0	207.2 ± 5.0	191.0 ± 15.2	164.8 ± 6.2
Total						
% change from previous process			-74.9	-2.7	-7.8	-13.7
% change from dry mix						-80.6
% change from dough						-22.6
P						
% change from previous process			-76.1	-6.6	-6.8	-16.5
% change from dry mix						-84.7
% change from dough						-27.3

^a mean ± standard deviation expressed as mg catechin equivalent/g as determined by normal phase HPLC; corrected for molecular weight

^b DP, degree of polymerization

^c DP > 10

^d ND, not detected

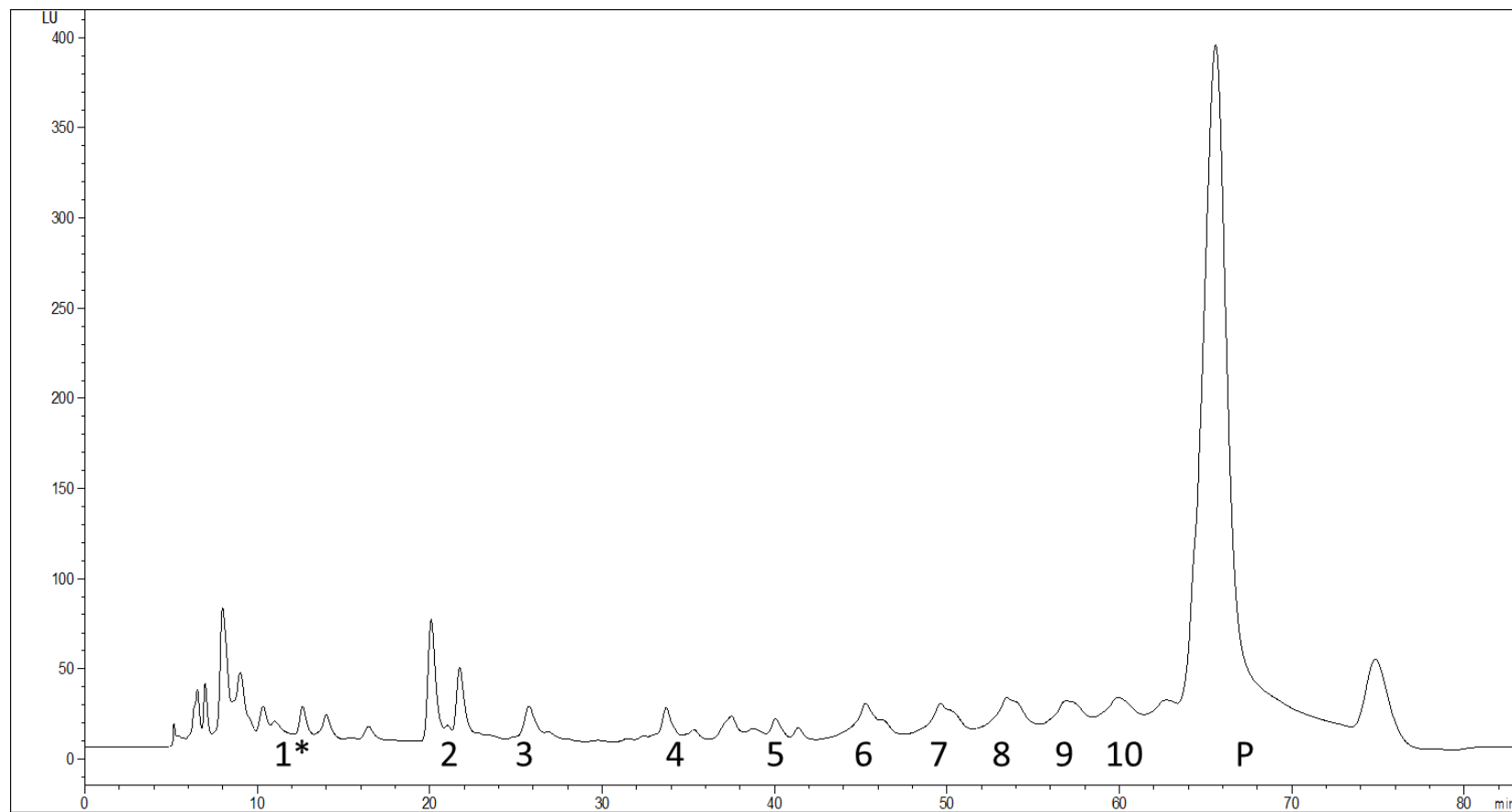


Fig. 15. Normal phase HPLC separation of proanthocyanidins in brown sorghum bran based on degree of polymerization (DP) (Ex=230, Em=321).

* DP; P = DP >10

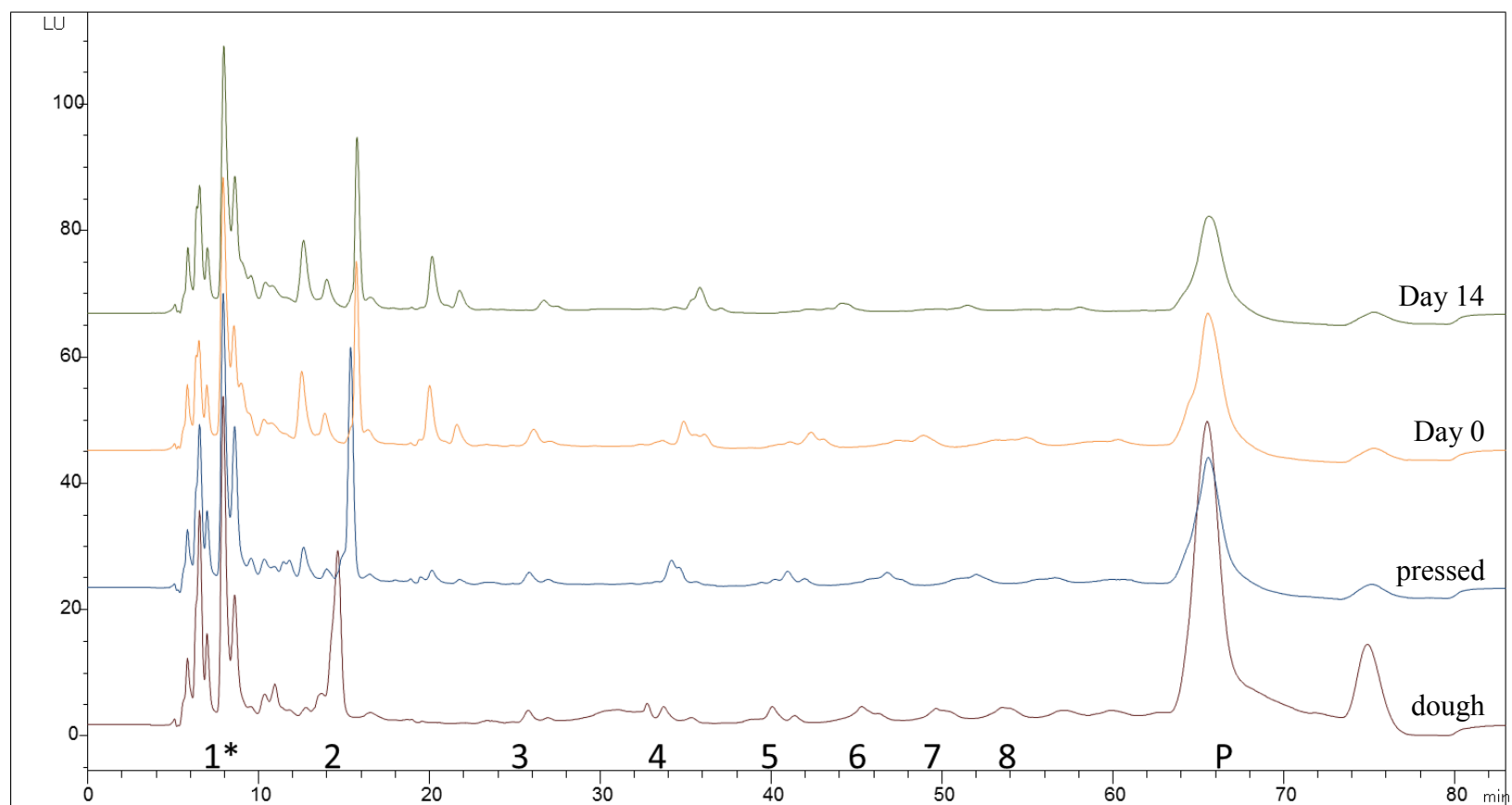


Fig. 16. Normal phase HPLC separation of proanthocyanidins based on degree of polymerization (DP) in dough, pressed, Day 0, and Day 14 tortillas prepared with brown sorghum bran at the 25% substitution level (40% fluorescence offset; Ex=230, Em=321).

* DP; P = DP >10

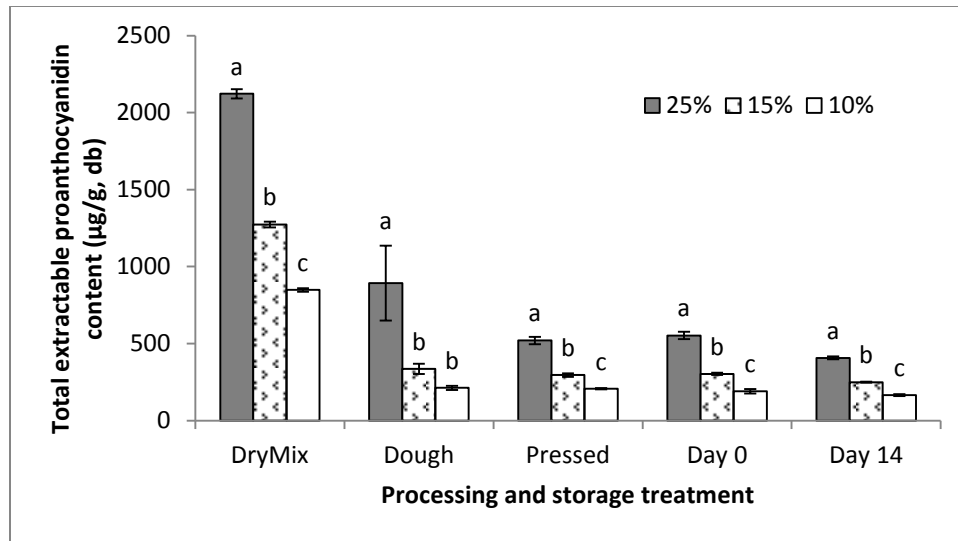


Fig. 17. Effect of processing and storage treatment on total extractable proanthocyanidin content (µg/g, db) of tortillas substituted with brown sorghum bran at 25, 15, and 10%, as determined by normal phase HPLC. Bars with the same letter within the same processing or storage treatment category are not statistically different ($p < 0.05$, LSD).

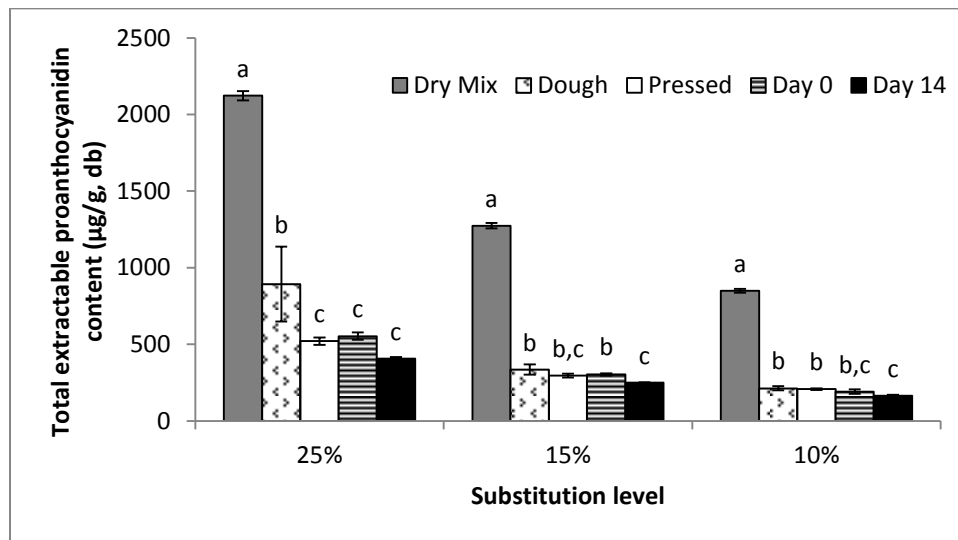


Fig. 18. Effect of brown sorghum bran substitution level on total extractable proanthocyanidin content (µg/g, db) of dry mix, dough, pressed, Day 0, and Day 14 tortillas, as determined by normal phase HPLC. Bars with the same letter within the same substitution level category are not statistically different ($p < 0.05$, LSD).

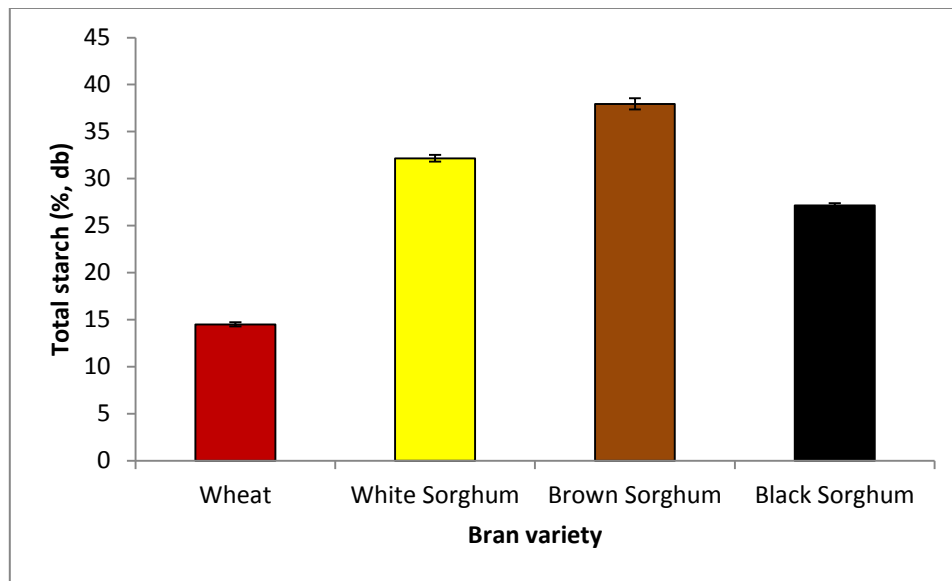


Fig. 19. Total starch (% db) of brans as determined by *in vitro* digestion.

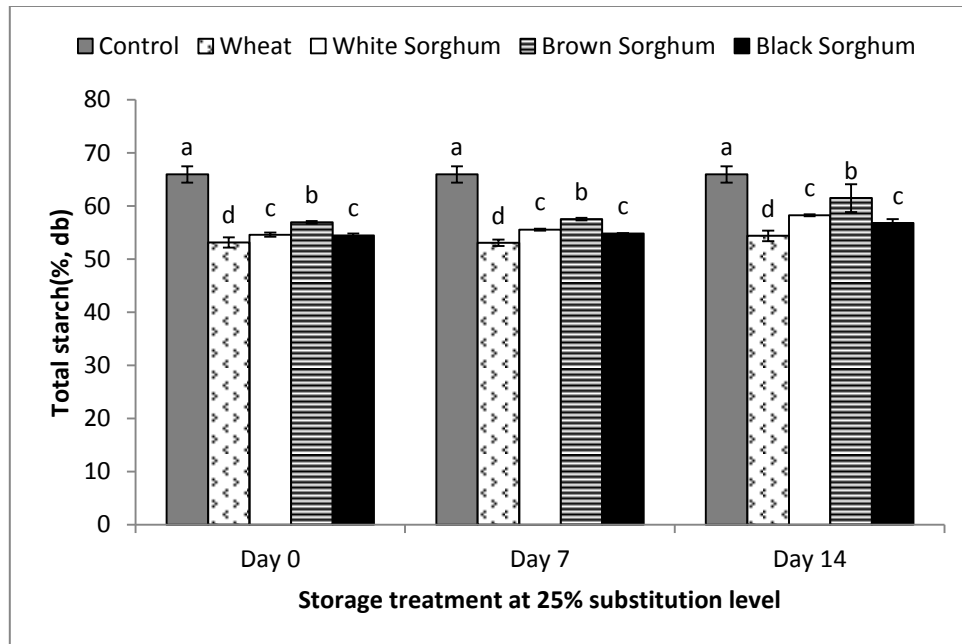


Fig. 20. Effect of storage time on total starch (% db) content of tortillas substituted at 25%. Bars with the same letter within the same storage treatment category are not statistically different ($p < 0.05$, LSD).

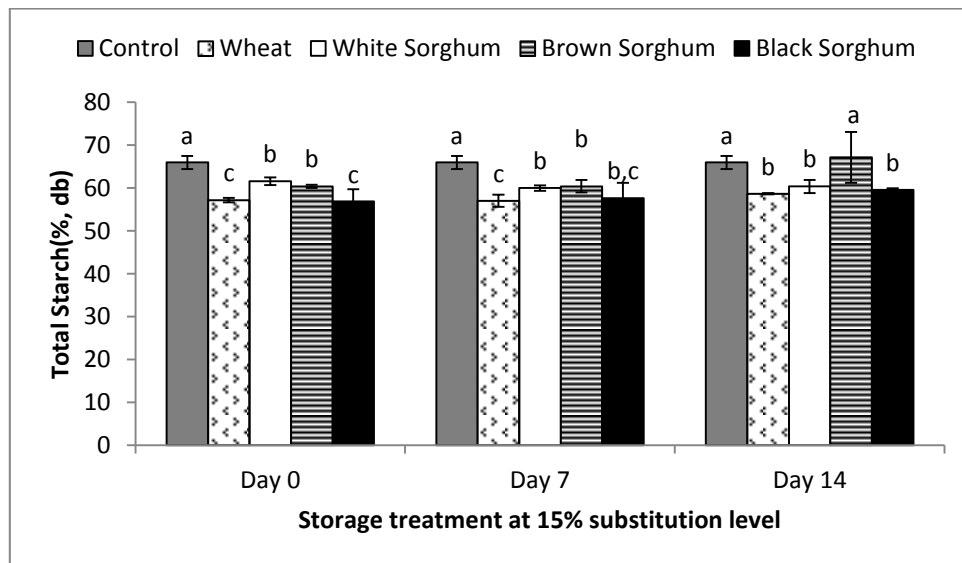


Fig. 21. Effect of storage time on total starch (% db) content of tortillas substituted at 15%. Bars with the same letter within the same storage treatment category are not statistically different ($p < 0.05$, LSD).

Effect of bran substitution on rapidly digestible (RDS) and slowly digestible starch (SDS)

The rate and extent of digestion of starch in the small intestine provide more information about the effect of a food on glycemic response than TS alone. RDS causes a rapid peak in blood glucose level as compared to SDS, which takes longer to digest and releases glucose over a prolonged amount of time (Lehmann and Robin, 2007). Foods that create a moderate glucose response have been shown to help manage blood glucose levels (Seal et al., 2005) and may contribute to satiety (Alvina and Araya, 2004; Sparti et al, 2000).

At the 25% substitution level, RDS content ranged from 81.24 – 88.95% in Day 0 tortillas, while SDS ranged from 11.05 – 18.76% (Figures 22 and 23). The tortillas prepared with brown sorghum bran had significantly lower RDS (81.24%) and significantly higher SDS (18.76%) than the other treatments at Day 0 ($p<0.05$) (Figures 22 and 23). In 14 day old tortillas, RDS and SDS ranged from 83.76 – 91.17% and 8.83 – 16.24%, respectively (Figures 22 and 23). Again, tortillas prepared with brown sorghum bran had the highest amount of SDS (16.24%), but were statistically similar to wheat (12.98%) and white sorghum (15.44%) bran tortillas ($p<0.05$) (Figures 22 and 23).

No significant differences in RDS or SDS were detected at the 10% and 15% substitution levels as compared to the refined flour control. No significant effect on RS was observed.

The effect of brown sorghum bran on RDS and SDS content suggests potential interactions of PA present in brown sorghum bran with starch that hinder digestion of starch. Analysis of phenolic content showed high molecular weight PA content decreased by 58% upon dough formation in brown sorghum bran substituted (25%) samples (Table IV), suggesting interactions occur during this processing step that contribute to the increase in SDS as compared to the refined control. High molecular weight PA have been shown to bind with starch to produce RS after processing of mixtures of pure starch and tannin extracts (Barros et al., 2012; Barros et al., 2014).

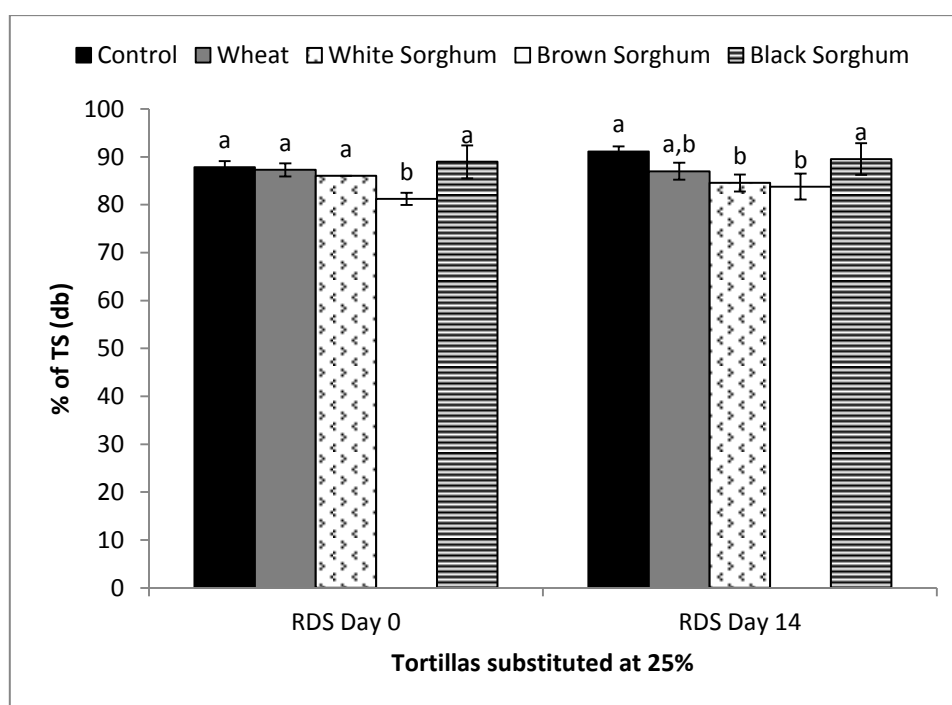


Fig. 22. Effect of bran source on rapidly digestible starch (RDS) (% of total starch, db) content of Day 0 and Day 14 tortillas substituted at 25%. Bars with the same letter within the same storage category are not statistically different ($p < 0.05$, LSD).

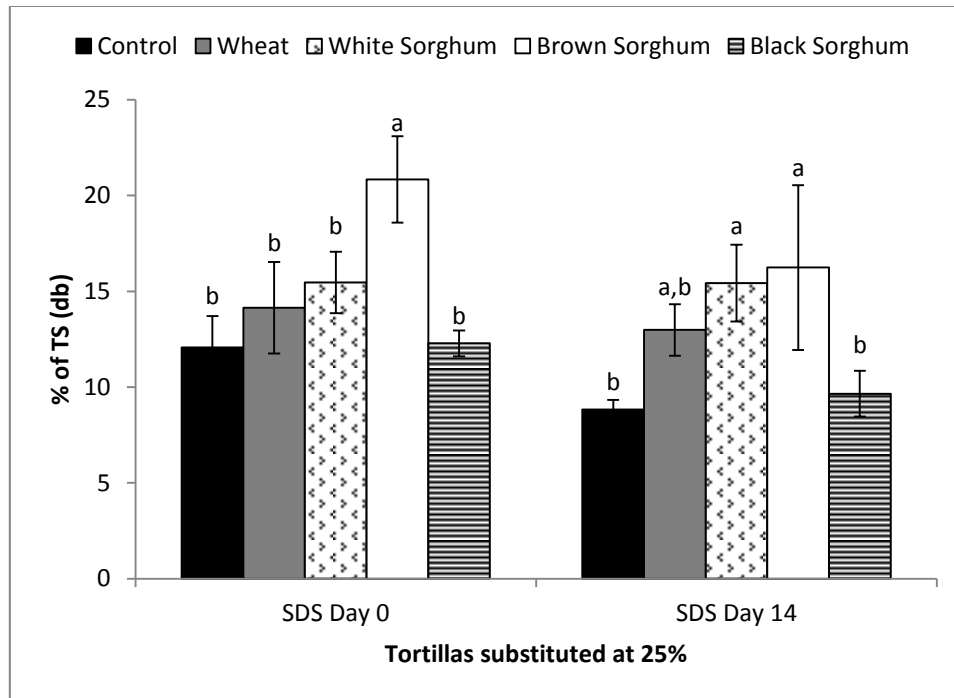


Fig. 23. Effect of bran source on slowly digestible starch (SDS) (% of total starch, db) content of Day 0 and Day 14 tortillas substituted at 25%. Bars with the same letter within the same storage category are not statistically different ($p < 0.05$, LSD).

Effect of bran substitution on total dietary fiber (TDF)

As with TS, the brans contained different levels of TDF (26.15 – 51.58%, db) (Figure 24). Bran substitution in tortillas increased TDF to 8 – 15% (Figure 25) as compared to 2%, which was the expected TDF in the refined control (Jondiko, 2010). TDF increased in tortillas prepared at the 25% substitution level after processing and 14 days of storage (output) as compared to the dry mix (input) (Figure 25). Sorghum bran increased TDF (21 – 26%) more than wheat bran (11%) (Figure 26). The increasing effect was observed for all sorghum brans, suggesting sorghum proteins contributed to the formation of dietary fiber. Sorghum proteins, specifically kafirins, cross-link upon

cooking to a greater extent than other cereal grains and decrease digestibility (Hamaker et al., 1986; Duodu et al., 2003; Guiragossian et al., 1978). Among sorghum brans, brown sorghum bran had the largest effect (26% increase) on TDF formation (Figure 26). PA interactions with starch and protein, in addition to protein cross-linking, may be responsible for the increase.

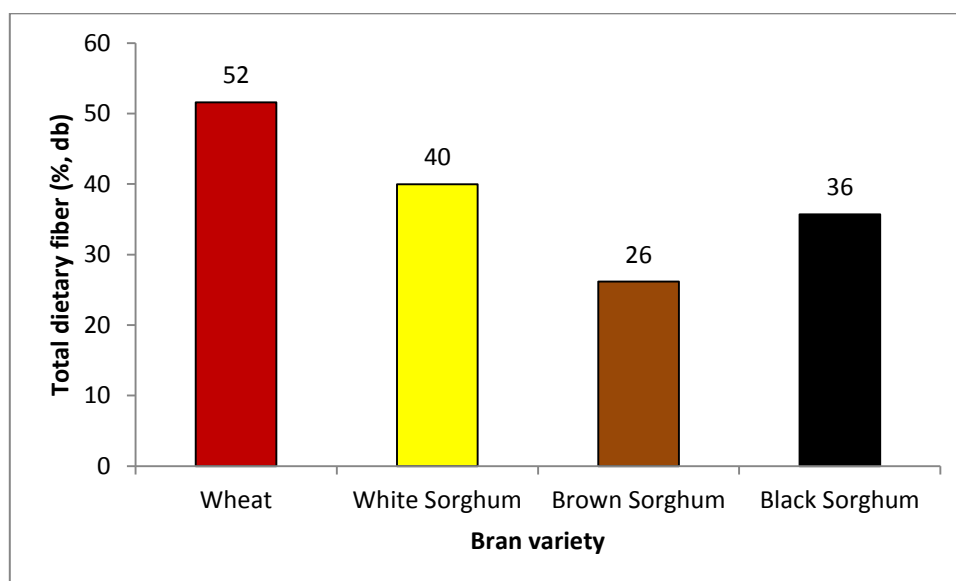


Fig. 24. Total dietary fiber (% db) of brans as determined by method 985.29 (AOAC Int., 2012).

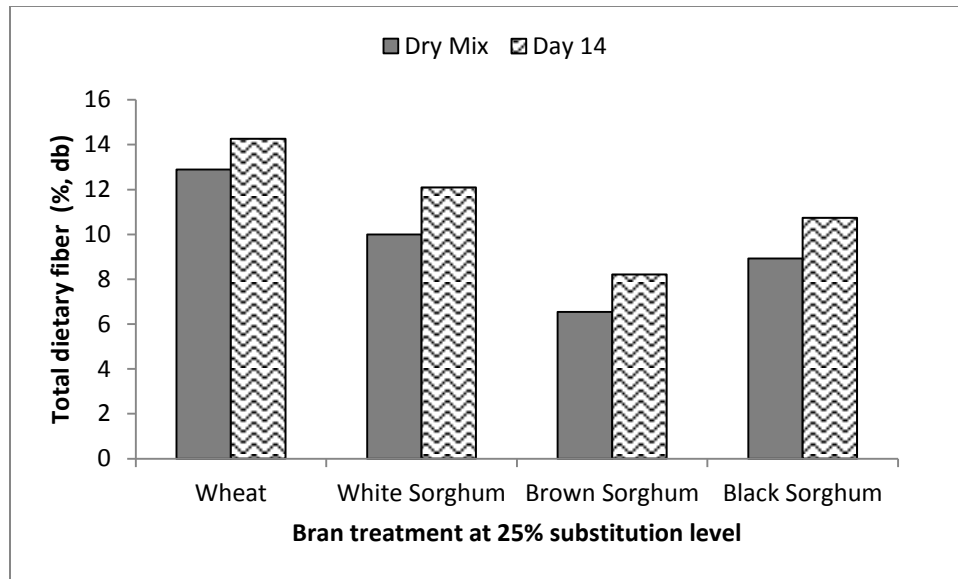


Fig. 25. Effect of bran source on total dietary fiber (% db) content of dry mix and Day 14 tortillas substituted at 25%.

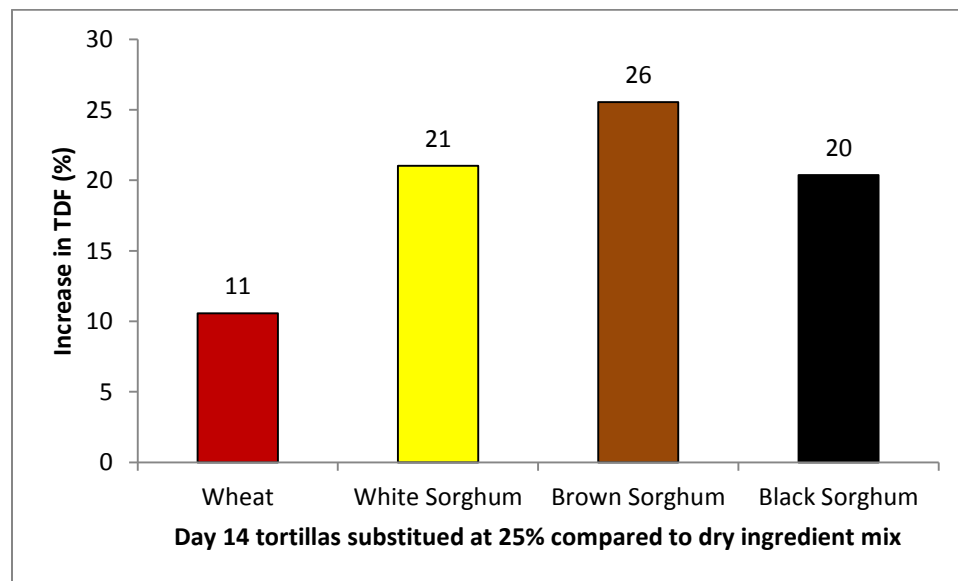


Fig. 26. Effect of bran source on percent increase in total dietary fiber (TDF) of Day 14 tortillas substituted at 25% as compared to TDF in the dry ingredient mix.

Chapter summary

Bran substitution increased force to compress and decreased stress relaxation time of tortilla dough; this produced dough that was more rough and stiff and less extensible and elastic as compared to the refined control.

Total phenol content in tortillas was significantly affected by bran source, dough formation, heat processing, and storage. Heat processing significantly decreased total phenol content across all samples. The largest decrease in total phenols was observed upon formation of dough in brown sorghum bran treatments with losses of 58 – 78%. Substitution of refined flour with brown and black sorghum brans at 15 and 25% may be used to significantly increase the total phenolic content of tortillas as compared to wheat and white sorghum brans.

Luteolinidin, 7-O-methyl-luteolinidin, apigeninidin, and 7-O-methyl-apigeninidin were the major 3-deoxyanthocyanin pigments identified in tortillas prepared with black sorghum bran. Similar trends in extractability and stability were observed for all four of the pigments and may have been affected by interactions with the protein matrix or retrograding starch. The stability of such pigments over processing and storage is essential to consider their use as natural food colorants, an increasing trend in the food industry.

The PA profile of brown sorghum bran tortillas shifted as a result of dough formation, processing, and storage of tortillas. Total extractable PA decreased with the largest proportion coming from high molecular weight PA while levels of monomers and dimers remained stable. The largest decrease in high molecular weight PA (58 – 76%)

was observed upon dough formation; this agrees with the large decrease in total phenols was also observed upon dough formation in the brown sorghum bran treatments. A decrease in extractable high molecular weight PA suggests they are participating in formation of insoluble complexes that are contributing to an increase in SDS and TDF.

When compared to other brans substituted at 25%, brown sorghum bran increased SDS by 35%. Higher levels of SDS and sustained blood glucose levels may trigger satiety and potentially benefit weight management and related diseases (Lehmann and Robin, 2007; Zhang and Hamaker, 2009). TDF in tortillas containing bran was higher (8 – 15%) than what was expected in the refined control (2%). Each bran treatment increased DF formation in tortillas; however, the increase in TDF was doubled in samples prepared with sorghum bran (20-26%) as compared to wheat (11%) at the 25% substitution level.

Large decreases in total phenols (58%) and high molecular weight polymeric PA (58%) in dough prepared with brown sorghum bran in addition to an increase in SDS (35%) and TDF (26%) of baked tortillas suggests condensed tannins are interacting with starch and protein to produce complexes that are not as readily digestible.

The hypothesis for this research was that polyphenolic compounds, specifically proanthocyanidins from brown sorghum, present in sorghum bran would interact with starch and slow down or prevent its digestion. This appears to be the case when looking at the data for SDS and TDF as brown sorghum bran tortillas exhibited the largest increase in those areas. However, the changes were relatively modest compared to what was expected based on previous observations using a pure starch model (Barros et al.,

2012). This suggests proteins in the bran and dough preferentially bind to the PA, limiting starch-PA interactions. In addition, increases were seen among all sorghum types as compared to wheat samples. This suggests other components present in all of the sorghum brans interacted with the tortilla matrix. Sorghum proteins exhibit cross-linking behavior when cooked, forming insoluble complexes that can affect availability of other nutrients (Hamaker et al., 1986; Duodu et al., 2003; Guiragossian et al., 1978; El Nour et al., 1998). Because of this, protein analysis is essential to determine if sorghum proteins participated in the interactions and contributed to the effects observed in this study.

CHAPTER III

EFFECT OF SORGHUM POLYPHENOLS ON PROTEIN PROFILE OF WHEAT FLOUR TORTILLAS

Introduction

Protein is an important functional component of baked goods. The amount and composition of protein in baked goods is important because it imparts eating qualities such as structural, rheological, and sensory attributes desired in the finished food product. In addition to providing these characteristics, protein also plays an important role in nutrition. Factors that influence protein functionality and bioavailability should be taken into consideration when producing processed foods.

Protein functionality is influenced by several factors. Intrinsic factors, such as composition, molecular weight, electric charge, and steric conformation, contribute to protein functionality. Proteins may also interact with other each other and form cross-linked complexes upon cooking. Cross-linking of the prolamine, or kafirin, fraction of sorghum has been associated with lower *in vitro* digestibility of sorghum proteins as compared to other grains (Hamaker et al., 1986; Duodu et al., 2003; Guiragossian et al., 1978).

Protein functionality in a food system is also dependent upon extrinsic factors such as pH, temperature, cooking process, and presence of non-protein compounds like starch and non-starch polysaccharides, polyphenols, and phytic acid that may interact with proteins (Duodu et al., 2003). For example, condensed tannins from sorghum have

been shown to bind with protein and reduce digestibility (Taylor et al., 2007; Asquith and Butler, 1986).

Physical interactions may simply block digestive enzyme activity whereas chemical interactions may render protein indigestible (Duodu et al., 2003). These interactions are also dependent upon the characteristics of the non-protein compound. For example, condensed tannins have a greater affinity for large, conformationally open proteins rich in proline residues (Butler et al., 1984).

When compared to commonly consumed grains such as corn and wheat, a limiting factor of sorghum for food use is the reduced digestibility of sorghum proteins after processing (Mertz et al., 1984; Axtell et al., 1981). However, interactions with non-protein compounds like starch and non-starch polysaccharides may slow down or inhibit starch digestion and reduce the caloric density of the food. This may be beneficial in prevention and management of obesity and associated diseases that are increasing in the United States.

Polyphenols from sorghum bran may preferentially bind proteins over starch in the wheat flour tortilla matrix; this would hinder the effect of sorghum polyphenols, specifically proanthocyanidins, on reducing the starch digestibility of the tortillas.

The objective of this work was to determine how polyphenolic compounds from sorghum bran affected the protein profile of wheat flour tortillas and the changes that occurred during tortilla processing and storage.

Materials and methods

Protein composition

Protein analysis was conducted in collaboration with Dr. Scott Bean (Grain Quality and Structure Research Unit, USDA-ARS, Manhattan, KS) on dough, pressed, and days 0 and 14 tortilla samples substituted at 25%.

Total protein determination

Nitrogen content of freeze-dried samples was determined by combustion using a LECO instrument (LECO Corporation, St. Joseph, MI) and converted to total protein by a factor of 5.7.

Extraction of soluble protein

The soluble protein fraction was determined as described by Schober et al (2006). Samples (100 mg) were extracted three times in 1 ml of 50% 1-propanol for 5 minutes. The samples were centrifuged after each extraction. The supernatant from the first two extractions was combined and used for size exclusion – high performance liquid chromatography (SE-HPLC) analysis while supernatant from the final extraction was discarded.

The sample remaining after extraction, or pellet, contained the insoluble protein (IP) fraction. The pellet was freeze-dried and its protein content was determined by combustion as mentioned previously. The difference in total protein and IP was used to calculate the amount of soluble, or extractable protein (EP), in the extracts.

Separation of soluble protein

The EP fraction in wheat is generally separated into three categories: soluble polymeric proteins (SPP), gliadins (GLI), and albumins and globulins (AG). SE-HPLC was used to separate the three fractions. The area of the peak for each fraction (as a percentage of the total peak area) was multiplied by EP to determine the amount (mg) of the respective soluble protein fraction. However, phenolic compounds eluted within the same time as the AG fraction in the brown and black sorghum bran treatments and interfered with the peak area estimation. This caused the AG peak area to be overestimated; therefore, the SPP and GLI were underestimated. To correct for this, SPP and GLI peak area were compared to the percent total protein and the AG data was not considered.

Statistical analysis

All procedures were performed in duplicate with the exception of total protein analysis when random check samples were duplicated. Means and standard deviations were determined using Microsoft Office Excel 2010 (Microsoft Corporation, Redmond, WA). Statistical Analysis Software (SAS) version 9.3 (SAS Institute, Cary, NC) was used for data analysis. Differences in treatments were determined at the 5% significance level ($\alpha=0.05$) using the general linear model (GLM) and least significant difference (LSD) for mean separation.

Results and discussion

Total protein

Total protein content of 14 day old tortillas substituted at 25% ranged from 11.4 – 14.1% (Figure 27). Subsequent protein results are reported as a percent of total protein.

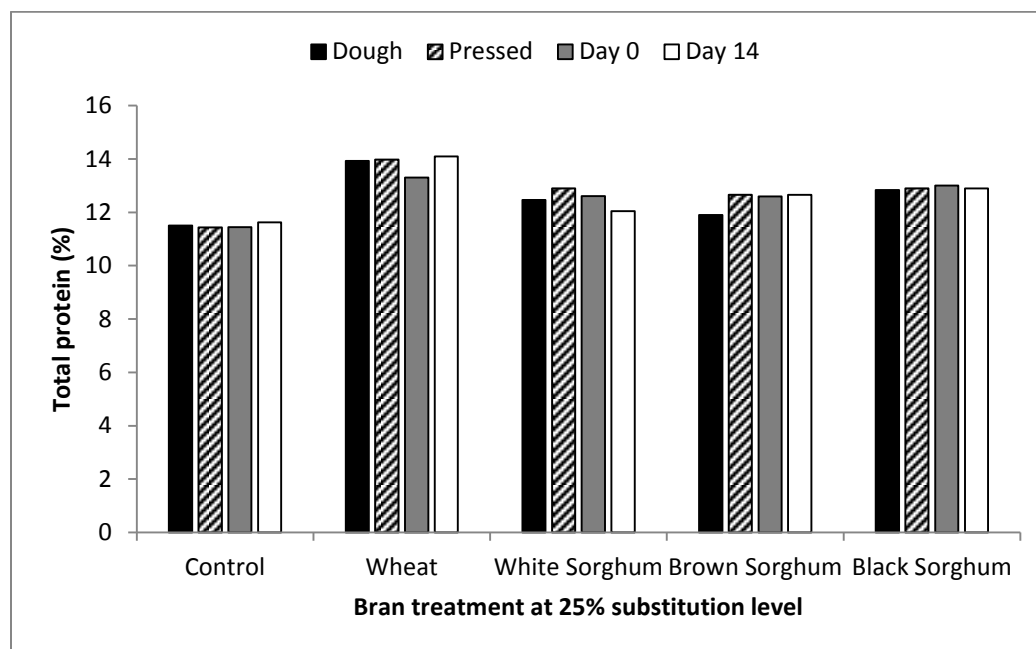


Fig. 27. Effect of bran source on total protein (%) content of dough, pressed, Day 0, and Day 14 tortillas substituted at 25%.

Insoluble protein (IP)

Molecular weight distribution influences protein functionality. IP is generally classified as high molecular weight insoluble glutenin polymers with a large high molecular weight (HMW) to low molecular weight (LMW) glutenin subunit ratio. However, IP may also include protein-polyphenol complexes. Increases in IP over

processing and storage indicate proteins are interacting with themselves or the tortilla matrix, such as starch or phenolic compounds, to produce insoluble complexes.

IP was significantly affected ($p < 0.05$) by bran source (Figure 28) as well as processing and storage (Figure 29). IP ranged from 33.0 – 45.6% in dough, and 34.5 – 47.2% in pressed, 51.2 – 58.4% in Day 0, and 51.5 – 60.1% in Day 14 tortillas. Dough and pressed samples prepared with white and black sorghum had the lowest amount of IP (Figure 28). However, the samples prepared with brown sorghum bran contained the highest levels of IP in dough (45.6%) and pressed (47.2%) tortillas (Figure 28). This sample also showed a 58% decrease in total phenols and a 58% decrease in high molecular weight proanthocyanidins (PA) upon dough formation. These results suggest insoluble protein-PA complexes are being formed as a result of interactions that occur during dough formation in the sample prepared with brown sorghum bran.

For all treatments, IP significantly increased after baking as compared to the dough and pressed product, but did not change after 14 days of storage ($p < 0.05$) (Figure 29). Tortillas prepared with brown sorghum bran also had the highest amount of IP after baking (58.4%) and storage for 14 days (60.1%), but were statistically similar to wheat and white sorghum bran ($p < 0.05$) (Figure 28). The Day 0 and 14 tortilla samples prepared with white and black sorghum bran were statistically similar to the wheat bran and control ($p < 0.05$) (Figure 28). Sorghum proteins, specifically kafirins (El Nour et al., 1997), have been shown to cross-link upon heat treatment and become insoluble (Hamaker et al., 1986; Duodu et al., 2002), contributing to the larger increase in IP of

white and black sorghum bran treatments after baking as compared to the control and wheat bran.

The increasing effect in IP of brown sorghum bran observed in the dough and pressed tortillas is not as obvious after baking. Proteins that would contribute to this effect due to cross-linking upon heat treatment may have already interacted in the dough formation step, making them unavailable to participate in cross-linking.

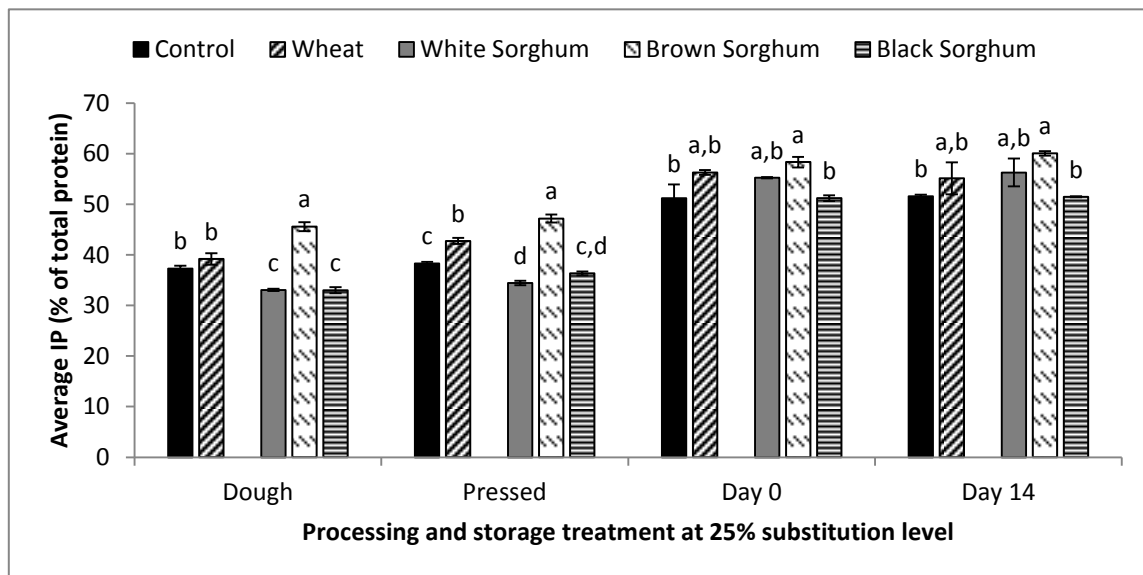


Fig. 28. Effect of bran source on insoluble protein (IP) (% of total protein) content of dough, pressed, Day 0, and Day 14 tortillas substituted at 25%. Bars with the same letter within the same processing or storage treatment category are not statistically different ($p < 0.05$, LSD).

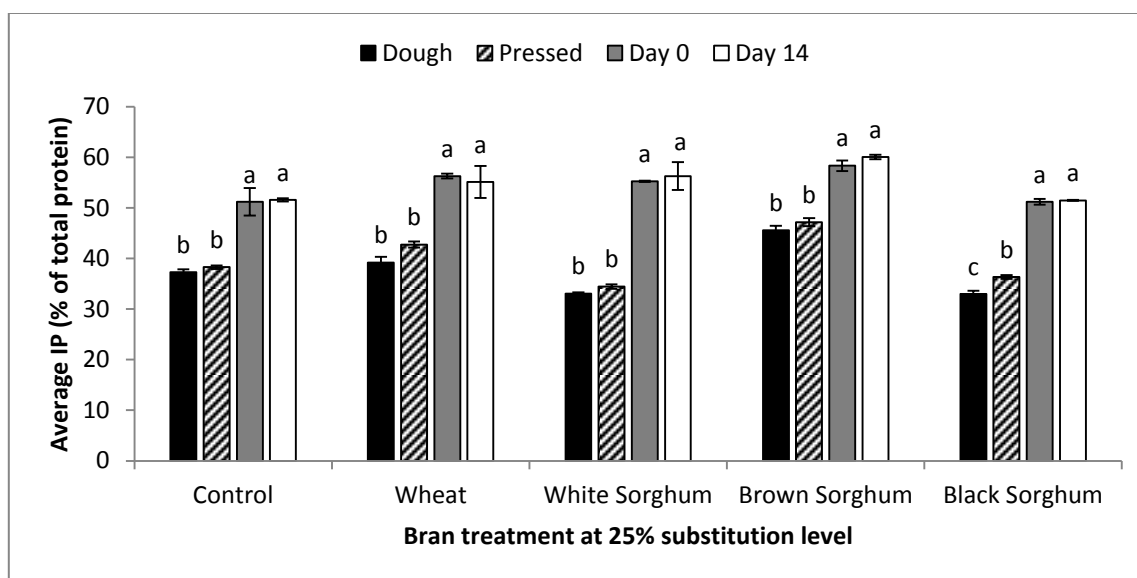


Fig. 29. Effect of processing and storage time on insoluble protein (IP) (% of total protein) content of tortillas substituted at 25%. Bars with the same letter within the same bran treatment category are not statistically different ($p < 0.05$, LSD)

Extractable protein (EP)

EP is inversely proportional to IP; therefore the opposite trends were observed in the EP results (Figures 30 and 31). Molecular weight influences affinity of proteins and PA. To determine if proteins of a certain molecular weight range were contributing to the observed increase in IP, EP was further separated into soluble polymeric proteins (SPP), gliadins (GLI), and albumin/globulin (AG) equivalents by SE-HPLC. SPP are soluble glutenin polymers with a continuous molecular weight range and may be classified as having a lower average molecular weight and lower HMW to LMW glutenin subunit ratio than IP. Compared to SPP, GLI are lower molecular weight monomers. AG are non-gluten proteins of a lower molecular weight than GLI (Schober et al, 2006).

Interference was detected in the AG peak area for the brown and black sorghum bran treatments, producing a larger peak area for AG. Therefore, the proportion of EP composed of AG was overestimated and SPP and GLI were underestimated in the brown and black sorghum bran treatments. To correct for this, levels of SPP and GLI are expressed as SE-HPLC peak area per percent total protein to account for differences in total protein and EP, respectively, and the AG peak area was not considered.

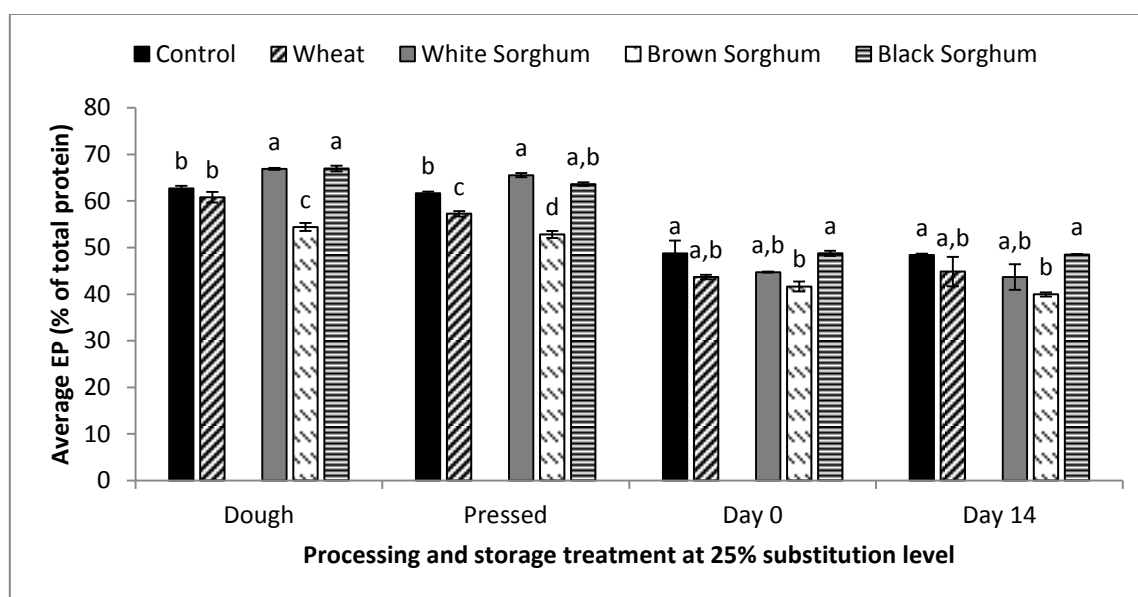


Fig. 30. Effect of bran source on extractable protein (EP) (% of total protein) content of dough, pressed, Day 0, and Day 14 tortillas substituted at 25%. Bars with the same letter within the same processing or storage treatment category are not statistically different ($p < 0.05$, LSD).

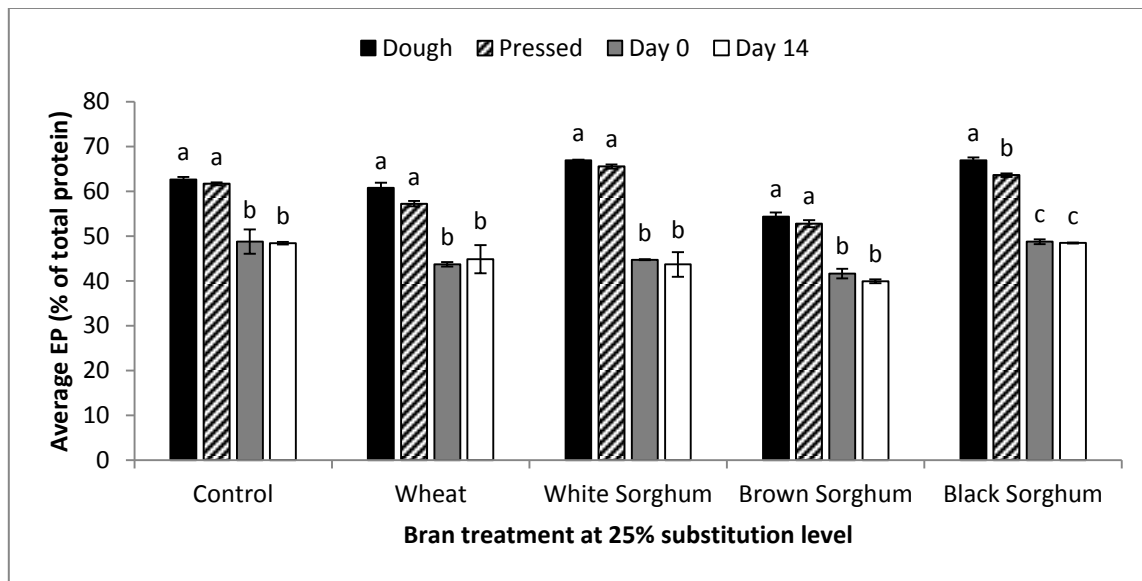


Fig. 31. Effect of processing and storage time on extractable protein (EP) (% of total protein) content of tortillas substituted at 25%. Bars with the same letter within the same bran treatment category are not statistically different ($p < 0.05$, LSD).

Soluble polymeric protein (SPP) and gliadins (GLI)

The largest changes in the EP fractions were observed in the SPP. For all treatments, SPP significantly decreased (40 – 61%) as a result of the baking process as compared to the dough ($p < 0.05$) (Figure 32); this supports the overall decrease observed in EP (Figure 31). Among sorghum brans, SPP in the brown sorghum bran treatment decreased the most (61%) from dough to Day 0 (Figure 32), agreeing with the trend observed in EP for sorghum bran treatments. This suggests that of the EP fractions, SPP interacts the most with PA to contribute to the observed increase in IP. Trends were less apparent for the GLI peak area (Figure 33), suggesting that the GLI fraction does not interact to form insoluble complexes.

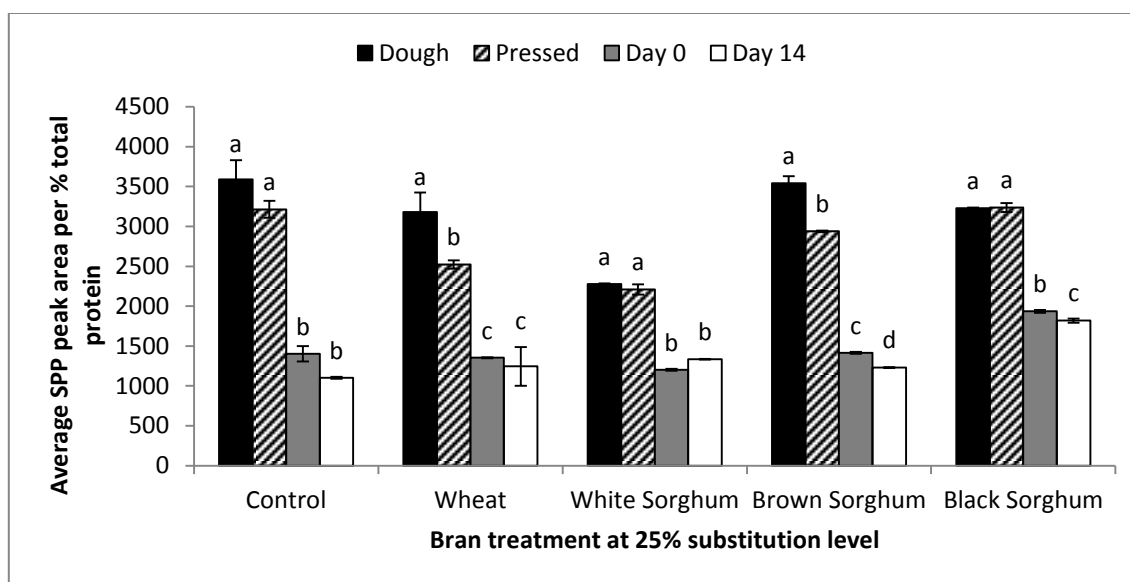


Fig. 32. Effect of processing and storage time on soluble polymeric protein (SPP) SE-HPLC peak area (per % total protein) content of tortillas substituted at 25%. Bars with the same letter within the same bran treatment category are not statistically different ($p < 0.05$, LSD).

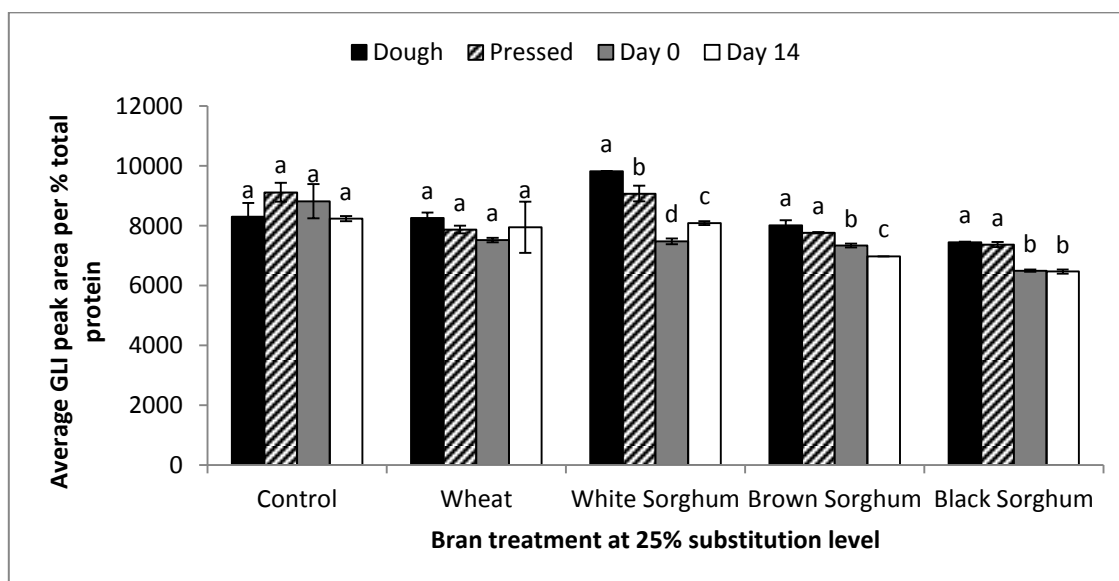


Fig. 33. Effect of processing and storage time on gliadin (GLI) SE-HPLC peak area (per % total protein) content of tortillas substituted at 25%. Bars with the same letter within the same bran treatment category are not statistically different ($p < 0.05$, LSD).

Chapter summary

The baking process increased IP and decreased EP across all bran treatments. The largest impact on IP was observed in dough and pressed samples prepared with brown sorghum bran, which had statistically higher amounts of IP ($p < 0.05$). Combined with previous data showing total phenol content and high molecular weight PA decreased as a result of dough formation, the increase in IP suggests proteins are forming insoluble complexes with condensed tannins present in the brown sorghum bran. Within the EP fraction, SPP significantly decreased after baking ($p < 0.05$).

Formation of protein-PA complexes and protein cross-linking with other protein bodies both contributed to formation of IP complexes; however, these two types of interactions may affect protein digestibility differently. Further work should investigate the effect of interactions between protein and phenolic compounds from sorghum on *in vitro* protein digestibility to determine if digestible protein is different from insoluble protein in these samples.

CHAPTER IV

CONCLUSIONS

Substituting refined wheat flour with sorghum bran affected the dough rheology, phenolic profile, starch digestibility, and protein extractability of wheat flour tortillas.

Tortilla dough produced with bran was rougher, more stiff and less extensible and elastic than the control prepared from refined wheat flour.

Processing and storage of tortillas significantly changed the phenolic profile and protein extractability of the tortillas. Overall, substitution with white sorghum bran produced results most similar to substitution with wheat bran. Brown and black sorghum bran substitution increased the total phenolic content of 14 day old tortillas two to fivefold as compared to wheat and white sorghum bran, respectively; therefore supplying more compounds that may act as antioxidants. As compared to black sorghum bran, total phenols decreased dramatically after dough formation in the brown sorghum bran treatments; this suggests that PA in the brown sorghum bran interacted with protein during the development of the dough protein network to form insoluble complexes.

Black sorghum bran also provided 3-deoxyanthocyanin pigments including luteolinidin, 7-O-methyl-luteolinidin, apigeninidin, and 7-O-methyl-apigeninidin. Extractability and stability followed the same trend for all of the major pigments at each level of substitution and was significantly affected by processing and 14 days of storage.

Changes in the molecular weight distribution of PA from brown sorghum bran were associated with increased SDS, TDF, and IP. The greatest effects of PA were

observed during tortilla dough formation. The largest changes were observed in PA with DP>10 (condensed tannins), which decreased by 58, 74, and 76% upon dough formation at the 25, 15, and 10% substitution levels, respectively, while monomers and dimers did not vary greatly over processing and storage. The large decrease in high molecular weight PA corresponds to the decrease observed in total phenols, suggesting insoluble condensed tannin-protein complexes were formed.

Changes in RDS and SDS were also greatest in samples prepared with brown sorghum bran. At the 25% substitution level, brown sorghum bran decreased RDS and increased SDS by 35% as compared to the other treatments. However, these results were lower than expected, suggesting that condensed tannins may preferentially bind protein over starch in the tortilla matrix. As compared to RDS, SDS is associated with better management of postprandial glucose response which is beneficial for consumers with diabetes. SDS may also contribute to satiety, potentially benefitting weight management and related diseases.

Bran substitution increased formation of TDF in 14 day old tortillas substituted at 25% when compared to the expected TDF. The largest increases were observed in the sorghum bran treatments (20 – 26%) and were twice as much of that observed in the wheat bran treatment (11%). Brown sorghum bran caused the greatest increase (26%). Sorghum proteins cross-link upon cooking and form insoluble complexes which may have contributed to the increase in TDF.

IP increased with baking and storage while EP decreased. Dough and pressed tortillas prepared with brown sorghum at the 25% substitution level had significantly

higher levels of IP ($p < 0.05$); this suggests insoluble complexes were formed during dough formation and supports the observed decreases in total phenols and extractable PA in the same treatment. When the EP fraction was further separated by SE-HPLC, it was observed that SPP decreased by 40 – 61% after baking, suggesting the SPP fraction was involved in interactions that contributed to the increase in IP after baking.

The changes in total phenols, extractable PA, and IP upon formation of dough prepared with brown sorghum bran, in addition to significant increases in SDS and greater TDF formation in baked tortillas suggest condensed tannins, protein, and starch interacted to form insoluble complexes that may provide beneficial health attributes compared to traditional refined wheat tortillas. A greater understanding of the role proteins play in formations of insoluble complexes with PA and their impact on macronutrient digestibility of tortillas as well as sensory profile of the tortillas are areas of further investigation.

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